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Growth and development of the mammary gland are controlled by the female hormones estrogen and progesterone. These hormones affect breast cancer growth and are targets for hormonal therapy. For this reason, it is important to understand the role of these hormones in the proliferation of mammary epithelial cells. We have characterized how these hormones may be interacting with the cell cycle machinery. The cell cycle machinery orchestrates the events required for cells to proliferate. It is composed of cyclins and their catalytic partners known as cyclin-dependent kinases. We have addressed how estrogen and progesterone affect expression and activities of these cell cycle molecules. We have shown that estrogen can induce cyclin D1 expression and activate cyclin E-cdk2 complexes in breast cancer cells. We have also investigated the relationship between progesterone and cyclin D1 using mice lacking either cyclin D1 or the progesterone receptor. Both strains have a defect in mammary gland development. We postulated that cyclin D1 may be regulated by progesterone during pregnancy. However, mammary glands lacking cyclin D1 responded to progesterone like normal mammary glands.

In conclusion, we have studied the role of estrogen and progesterone in regulating the cell cycle of mammary epithelial cells. Understanding the growth-regulatory mechanisms affected by these hormones may lead to new ways of breast cancer therapy.

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INTRODUCTION

The projects described here are designed to elucidate the molecular mechanisms that regulate the proliferation of mammary epithelial cells in mice and in humans. These cells are the targets of oncogenic conversion during the processes that lead to breast carcinomas in these species. Because of the very similar biology of human and mouse mammary tissues, the latter can be used to model certain aspects of the biology of the human breast.

The present experiments address two major issues relating to the proliferative control of mammary tissue. First, the morphogenesis of breast tissue is highly complex, being regulated by a number of distinct morphogens that influence distinct phases of mammary epithelial morphogenesis including ductal extension, ductal branching, and the formation of alveoli. We surmise that certain of these steps run awry during tumor pathogenesis and accordingly are attempting, as described here, to elucidate these morphogenetic steps as they occur within the normal breast tissue. Second, estrogen is a known mitogen for a substantial proportion of human mammary carcinomas, yet the precise biochemical mechanisms by which the estrogen receptor activates the proliferative machinery of mammary carcinoma cells has remained elusive. The second of the projects described here addresses the mechanism by which the estrogen receptor activates the cell cycle clock apparatus of human MCF-7 mammary carcinoma cells.

BODY

Project 1: Hormonal control of lobuloalveolar proliferation

Our experiments addressing the development of the mammary tissue began with our observation that mice lacking the cyclin D1 gene fails to lactate because of a defect in the proliferation of their mammary epithelium(**1**). This developmental lesion was highly specific. Thus, while the mammary ducts develop properly prior to pregnancy in these mice, lobuloaveolar development in response to pregnancy fails to occur. A superficially similar phenotype has been reported by Lydon et al., who have described the developmental defects in mice associated with the germline inactivation of the progesterone receptor (PR) gene(**2**). As was the case with the cyclin $D1^{-/-}$ mice, ductal formation seemed to occur properly while lobuloalveolar development was minimal. Some of our experiments over the past year have been directed toward understanding the relationship between the developmental defects of these two strains of mice.

a. Analysis of mice lacking the progesterone receptor

Mice carrying an inactivated PR gene in their germline were provided to us through a collaboration with Drs. John Lydon and Bert O'Malley. The initial analysis of the breast-specific developmental defects in the laboratory of these other workers was limited: since the $PR^{-/-}$ mice fail to become pregnant, the responsiveness of their mammary epithelium to pregnancy-associated hormones was difficult to ascertain. In response to this, our collaborators introduced ectopic progesterone and estrogen into these mice in order to mimic the hormonal environment of pregnancy and failed to observe appropriate lobuloalveolar development(**2**). However, such observations are difficult to interpret, since these ectopically introduced hormones fail to recapitulate accurately the complex hormonal state of normal pregnancy.

We therefore designed transplantation experiments in which the effects of the hormonal environment of pregnancy on the $PR^{-/-}$ mammary epithelium could be properly assessed(**3**). In particular, we used females of the RAG1 strain to accept $PR^{-/-}$ and wild type mammary grafts. These mice are immunocompromised and hence are able to accept grafts from mice of various strains; however, in contrast to nude mice, which do not nurse their pups, these mice have normal reproductive functions(**4**).

When $RAG1^{-/-}$ females were engrafted with $PR^{-/-}$ mammary epithelium and then induced to become pregnant, their ductal branches formed normally but were unable to produce the normal levels of sidebranching and subsequently to sprout alveoli. Importantly, this developmental block seemed to occur an earlier stage in development than was seen with the cyclin D1-negative mice. The latter were able to form sidebranches properly but did not form alveoli(**3**).

These observations provided no insight into the target tissue in the breast that requires PR in order for mammary development to proceed properly. Because the PR is expressed to some extent in both the stroma and epithelium(**5**), it was unclear what the primary and direct target of progesterone was in this organ. In response to this, we created mixed grafts, in which wild type epithelium was implanted together with $PR^{-/-}$ stroma or conversely, in which $PR^{-/-}$ epithelium was implanted with wild type stroma. In both cases, these reconstituted mammary tissues were engrafted into $RAG1^{-/-}$ females, which were then allowed to become pregnant.

The outcome of these experiments was clear and unambiguous. The presence of the PR in the stroma (i.e. fat pad) had no effect whatsoever on the mammary development. Conversely, the PR needed to be present in the mammary epithelial cells (MECs) forming the epithelium in order for mammary development to proceed normally(**3**).

We then wished to narrow further the site of action of the PR within the mammary epithelium. In particular, it was unclear whether the PR needed to be expressed in all MECs or only in a subset of the MECs to allow sidebranching and lobuloalveolar development to occur. To address this problem, we created mixed populations of PR-positive and -negative MECs which were then implanted into a cleared, wild type fat pad.

The goal in this experiment was to ascertain whether the presence of the PR in a subset of MECs would suffice to permit lobuloalveolar development to occur appropriately. This required our ability to distinguish between the $PR^{+/+}$ and $PR^{-/-}$ MECs in the engrafted breast tissue. We did so by using MECs derived from mice of the ROSA26(**6**) strain, which express the enzyme ß-galactosidase in all of their tissues, rendering their cells blue upon staining with an appropriate substrate indicator. This allowed us to analyze engrafted chimeric breasts and to identify the genetic origin of the various cells in the chimeric tissue. In the absence of the ß-galactosidase enzyme, MECs appeared red upon counterstaining, allowing us therefore to distinguish between red and blue MECs.

When we cointroduced $PR^{-/-}$ MECs together with $PR^{+/+}$ ROSA cells into the fat pads of RAG1 females, we found both red and blue cells in the alveoli that appeared when these females became pregnant. We concluded tentatively that the presence of the PR was not essential in the MECs of alveoli in order for these alveoli to develop normally, at least when the $PR^{-/-}$ MECs were in close proximity to $PR^{+/+}$ cells(**3**).

Nonetheless, this experiment was susceptible to artifact, as it remained possible that the cells that stained red in the alveoli (and were therefore ostensibly $PR^{-/-}$) were actually $PR^{+/+}$ MECs in which, for unknown reasons, the ß-galactosidase staining reaction had failed to go to completion. To control for this possibility, we crossed $PR^{-/-}$ mice with those of the ROSA strain in order to generate $PR^{-/-}$ MECs that expressed the ß-galactosidase transgene. These were then co-introduced together with $PR^{+/+}$ MECs that lacked the ß-galactosidase marker into wild type fat pads.

The results of these experiments were similar to those seen before. On this occasion, we found blue as well as red alveoli, proving that $PR^{-/-}$ MECs can participate directly in alveolar formation. However, the blue $PR^{-/-}$ alveoli were always seen in close apposition to red ducts. From this observation, we draw the conclusion that the PR must be present in MECs of the ducts, and that the latter, in response to progesterone, release a paracrine signal that enables nearby MECs to initiate lobuloalveolar development(**3**). This paracrine signal must, we presume, act over very short distances between the $PR^{+/+}$ MECs that respond to progesterone directly and the nearby $PR^{-/-}$ MECs that form the alveoli. The nature of this paracrine signal remains unknown.

b. Analysis of defects in hormone responsiveness of MECs that lack cyclin D1

In parallel with the work described above, we returned to the cyclin $D1^{-/-}$ in order to understand their developmental defect more precisely. Experiments performed last year had shown that when $D1^{-/-}$ MECs were engrafted into cleared fat pads of cyclin D1 wild type hosts, these MECs failed to develop alveoli in response to pregnancy(**7**). We concluded from this that the defect in the $D1^{-/-}$ cells was indeed intrinsic to these cells and was therefore a cell-autonomous defect. Conversely, we could rule out the possibility that the mammary defect in the $D1^{-/-}$ mice was due to some type of systemic endocrine defect in these mice.

We refined this analysis by determining the responsiveness of $D1^{-/-}$ cells to progesterone and to prolactin, two of the prominent pregnancy-associated hormonal morphogens. Both the wild type and $D1^{-/-}$ mammary epithelia responded to ectopically introduced progesterone by developing sidebranches in the mammary epithelium, indicating that the progesterone-dependent step in mammary development precedes and is not affected by the cyclin D1-dependent step(**7**). This observation was in consonance with earlier observations, mentioned above, which indicated that the PR-dependent step of mammary morphogenesis precedes the step that depends upon cyclin D1.

We also analyzed the effects of prolactin on the wild type and cyclin $D1^{-/-}$ epithelia. As before, we used the mammary fat pads of RAG1 females as the recipients of the various grafts. Three weeks after initial engraftment of the wild type and the mutant MECs, we introduced pituitary grafts under the kidney capsules of the engrafted host animals. The engrafted pituitaries produce a steady stream of prolactin(**8**), since the engrafted pituitary is no longer under the control of hypothalamus which normally represses its synthesis; other pituitary-specific hormones are no longer made by the ectopically engrafted pituitary gland.

Analysis of a number of such engrafted animals demonstrated that the wild type epithelium responded to the prolactin by producing alveoli while the mutant $D1^{-/-}$ epithelium failed to do so. This represents an important step forward for us, in that it suggests the possibility that the morphogen that is responsible for alveolar morphogenesis to which cyclin $D1^{-/-}$ epithelium can not respond is prolactin. This has now provoked us to begin an examination of the biochemical connection between the prolactin receptor and the cyclin D1 gene transcriptional promoter(**9**).

Project 2: Control of the cell cycle clock by the estrogen receptor

Estrogen is an important morphogen during puberty and pregnancy. However, its precise role in the complex morphogenetic steps that occur in the mammary epithelium is poorly understood. Indeed, the identity of the target cells in the normal epithelial ductal tree and/or alveoli remains elusive. Nonetheless, it is clear that estrogen assumes a central role in driving the proliferation of more than half of the mammary tumors that are diagnosed in humans(**10**). This leaves open two possibilities. These tumors may arise from a still-to-be-identified subtype of MEC that is normally responsive to estrogen and is driven to proliferate by this steroid

hormone. Alternatively, during the multi-step progression that leads to mammary carcinomas, premalignant MECs acquire the ability to exploit ambient estrogen as a mitogen, perhaps by altering the normal signal transduction pathways that usually connect the estrogen receptor (ER) with the cell cycle clock apparatus. In order to explore these possibilities, we have spent the last two years defining with precision the connections between the ER and the cell cycle clock in cells of the MCF-7, estrogen-responsive human mammary carcinoma cell line.

Initial observations demonstrated that we could synchronize MCF-7 cell cultures by blocking their proliferation with tamoxifen, the estrogen antagonist, and then reversing this block by introducing estrogen and removing tamoxifen. This led to subsequent synchronous entry into S phase. We were, however, more interested in the events that occur during the G1 phase following reversal of the tamoxifen-imposed block.

Detailed examination of cell cycle clock components in these synchronized MCF-7 cells revealed that while the activity of the cyclin E:CDK2 kinase increased synchronously 10-20 fold prior to S phase entrance, the levels of the two components of this complex, cyclin E and CDK2, did not change significantly. Cyclin D1 synthesis increased reproducibly threefold; often its synthesis was induced by a substantially greater amount.

These observations caused us to ask how the cyclin E:CDK2 complexes were inhibited during the period of the tamoxifen block. We used in vitro enzyme assays for the cyclin E:CDK2 to demonstrate that the tamoxifen-blocked cells accumulated a soluble inhibitor of CDK2 kinase activity, the levels of which decline precipitously upon estrogen stimulation of growth. Using antisera that are specific for various CDK inhibitors, we found that immunodepletion of the p21 CDK inhibitor removed the soluble inhibitor of cyclin E:CDK2 complexes that accumulated in the tamoxifen-blocked cells(**10**).

This provoked the further question of the mechanism by which estrogen stimulation caused the depletion of soluble p21 from MCF-7 cells. One attractive model was that estrogen causes the increase in cyclin D1; the resulting cyclin D1:CDK4/6 complexes bind increasing amounts of p21, thereby depleting it from the soluble pool and ultimately abstracting it from cyclin E:CDK2 complexes. When the cyclin D1:CDK4/6 complexes begin to exceed the levels of p21, then these cyclin D1 complexes become activated, which in turn makes possible the initial phosphorylation of pRB, the retinoblastoma protein. This sequestration of p21 then enables the activation of cyclin E:CDK2 complexes, which proceed to complete the phosphorylation of pRB, thereby ushering the cell through the mid/late G1 restriction point into late G1 and ultimately S phase.

We have now tested and validated several predictions of this mechanistic model, which we feel accurately describes the molecular events that make possible the estrogen-stimulated growth of the MCF-7 cells. Thus, we have found that estrogen stimulation causes a redistribution of p21 from cyclin E:CDK2 complexes to cyclin D1:CDK4/6 complexes. Indeed, this redistribution closely precedes the activation of the cyclin E:CDK2 complexes(**10**).

Interestingly, this redistribution of p21 still left the majority of cyclin E:CDK2 molecules bound to either p21 or to the other important CDK inhibitor in these cells, p27. Nonetheless, the resulting free cyclin E:CDK2 complexes apparently suffice to drive cells into late G1. For example, when we analyze the catalytically active cyclin E:CDK2 that is formed in late G1 following estrogen stimulation, we find these complexes lack both p21 and p27 subunits.

Our work has also ruled out other alternative mechanistic models. For example, it was possible that the inactivity of the cyclin E:CDK2 complexes in tamoxifen-treated cells was due to the presence of inhibitory phosphates that were attached to the CDK2 molecules. These phosphates are normally removed by the CDC25A phosphatases which in this way act as agonists of G1 progression. Hence, we tested whether the inactive CDK2 complexes found in tamoxifen-treated cells were activatable in vitro by addition of recombinant CDC25A. Treatment with this phosphatase failed to activate the latent cyclin E:CDK2 complexes found in the tamoxifen-treated cells. We therefore concluded that other mechanisms, notably the presence of the p21 inhibitor as described above, were responsible for the inactivity of the cyclin E:CDK2 complexes(**10**).

These results have stimulated us to determine whether estrogen is in general capable of inducing cyclin D1 synthesis in mammary epithelial cells, or whether this is a peculiarity of the MCF-7 cells and similar tumor cells. To this end, we have introduced expression vector specifying the ER into ER-negative mammary tumor cells and are currently testing these transfectants for the inducibility of their cyclin D1 genes by estrogen.

To date, we have failed to induce cyclin D1 synthesis. We are therefore following up two possibilities. First, that the cyclin D1 levels in these cells are already induced to maximum levels by other mechanisms including notably an activated RAS-RAF-MAPK-AP1 pathway. Second, that the ER is unable to act on the cyclin D1 promoter of these cells, perhaps because it does not associate with the appropriate array of transcriptional co-activators.

These results, while preliminary, have also stimulated us to examine the ability of estrogen to elicit cyclin D1 synthesis in normal mouse and human MECs and in a variety of tumor cells lines. This research has not yet yielded any results of substance. Nonetheless, it promises to reveal whether this connection between the ER and cyclin D1 represents a normal physiologic signalling pathway or an invention of mammary tumor cells that create pathway and exploit available estrogen to drive their own proliferation.

CONCLUSIONS

The work reported here allows us a number of conclusions concerning the mechanisms regulating the proliferation of mammary epithelial cells within the normal breast tissue and in vitro.

1. The progesterone receptor required for normal mammary development must be expressed in mammary epithelial cells; its expression in the mammary stroma is unnecessary.

2. In order for normal mammary development to proceed, the progesterone needs to be expressed only in a subset of mammary epithelial cells.

3. In order for lobuloalveolar development to proceed normally, the progesterone receptor needs to be expressed in ductal cells that are found in close apposition to an alveolus but needs not be expressed in the cells of the alveolus itself.

4. The requirement for cyclin D1 expression for normal lobuloalveolar development is intrinsic to the mammary epithelial cells. Thus, cyclin D1 does not need to be expressed in other cell types of the breast or in other organs in order for lobuloalveolar development to proceed properly.

5. The progesterone-induced ductal sidebranching in mammary development precedes and is not dependent upon the actions of cyclin D1.

6. In the mammary carcinoma cell line, MCF-7, the estrogen receptor is capable of directly activating expression of cyclin D1.

7. The activation of cyclin D1 by the estrogen receptor causes in turn an increase in cyclin D1:CDK4 complexes. These in turn abstract the p21 CDK inhibitor away from cyclin E:CDK2 complexes, allowing the latter to become activated, and permitting progression of the cell into late G1 and S phases.

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APPENDICES

Included herein are two scientific papers, one of which has been published and the other of which is in preparation.

Planas-Silva, M.D., and Weinberg R.A. (1997) Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. Molecular and Cellular Biology, 17:4059-4069.

Cathrin Brisken, Tibor Vass, Sissela Park, John P. Lydon, Bert W. O'Malley, and Robert A. Weinberg (1997) The Role of the Progesterone Receptor in Mammary Gland Development.

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Estrogen-Dependent Cyclin E-cdk2 Activation through p21 Redistribution

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In order to elucidate the mechanisms by which estrogens and antiestrogens modulate the growth of breast cancer cells, we have characterized the changes induced by estradiol that occur during the G_1 phase of the cell cycle of MCF-7 human mammary carcinoma cells. Addition of estradiol relieves the cell cycle block created by tamoxifen treatment, leading to marked activation of cyclin E-cdk2 complexes and phosphorylation of the retinoblastoma protein within 6 h. Cyclin D1 levels increase significantly while the levels of cyclin E, cdk2, and the p21 and p27 cdk inhibitors are relatively constant. However, the p21 cdk inhibitor shifts from its association with cyclin E-cdk2 to cyclin D1-cdk4, providing an explanation for the observed activation of the cyclin E-cdk2 complexes. These results support the notion that cyclin D1 has an important role in steroid-dependent cell proliferation and that estrogen, by regulating the activities of G_1 cyclin-dependent kinases, can control the proliferation of breast cancer cells.

A variety of models have been proposed to explain how estrogen drives the proliferation of normal mammary epithelial cells and breast cancer cells (8). By acting through the estrogen receptor (ER), estrogen can regulate the transcription of a cohort of responsive genes and in this way appears to regulate cell cycle progression. Even in the face of still-incomplete mechanistic insight into how estrogen regulates growth, effective antitumor therapies directed against the ER have been developed around the use of antiestrogens such as tamoxifen (20). Many studies of the effects of tamoxifen have indicated that tamoxifen acts in a cytostatic fashion on breast cancer cells, causing them to arrest in the G_0/G_1 phases of their growth cycle (36, 49). For these reasons, it is important to understand how estrogens and antiestrogens control G_1 progression.

The central regulator of this process is the cell cycle clock apparatus, which operates in the cell nucleus and is assembled from an array of cyclins and cyclin-dependent kinases (cdks) (45). The activities of the cdks are positively controlled by their association with cyclins and restrained by cdk inhibitors. Included among the latter are p21, p27, and p57, which can inhibit a wide range of cyclin-cdk complexes, and the INK4 family (p15, p16, p18, and p19), which specifically inhibits cdk4 and cdk6 (46).

Extracellular signals such as those conveyed by growth factors affect the activity of cyclins and cdks largely during the G_1 phase of the cell cycle. The most important components of the cell cycle clock apparatus during this period are (i) the D-type cyclins together with their catalytic partners cdk4 and cdk6 and (ii) cyclin E, which interacts with cdk2. Both classes of G_1 cyclin-cdk complexes are known to drive the phosphorylation of the retinoblastoma protein (pRb) (18, 19, 21, 42). This phosphorylation represents a key event in G_1 progression (53). Hypophosphorylated pRb is active in mediating G_1 arrest while hyperphosphorylated pRb appears to be inactive in blocking cell cycle advance.

Overexpression and amplification of G_1 cyclin genes have been observed in a number of primary breast cancers and in tumor-derived cell lines (7, 23, 24). For example, amplification of the chromosomal region 11q13 containing the cyclin D1 gene is frequently observed in breast cancer (26). This amplification seems to occur preferentially in ER-positive tumors and has been linked to poor prognosis (1, 44). Consistent with an important causal role in breast cancer, cyclin D1 overexpression can be observed in primary breast cancers, even at early stages of the disease (3, 54). Microinjection of antibodies or antisense to cyclin D1 during G_1 can prevent cell cycle progression of pRb-positive breast cancer cell lines (3). Moreover, ectopic expression of the cyclin D1 gene in the breast cancer cell line T47D shortens G_1 and induces cell cycle progression (33). Overexpression of cyclin D1 in breast cancer cells also reduces their rate of exiting from the cell cycle, allowing cell cycle progression and pRb phosphorylation even in the absence of growth factors (56).

Other research using mouse models has supported the notion that cyclin D1 plays a central role in regulating the proliferation of mammary epithelial cells. Transgenic mice expressing cyclin D1 under the control of the mouse mammary tumor virus promoter develop mammary hyperplasias and carcinomas in a pregnancy-dependent fashion (51). Moreover, the mammary glands of mice lacking the cyclin D1 gene fail to undergo full development during pregnancy while virtually all other tissues in these mice develop normally (12, 47). Since the main extracellular regulators of mammary development are ovarian steroids, the above results strongly support the notion that cyclin D1 is involved in mediating the steroid-dependent growth of mammary epithelial cells.

Indeed, several reports have suggested a role for steroids in regulating cyclin D1 expression. Musgrove et al. (32) were able to associate progesterone-dependent G_1 progression with changes in the expression of cyclin D1. Work from the same laboratory has suggested that the main target of antiestrogen action is cyclin D1 (52). Treatment of ER-positive breast carcinoma cell lines with antiestrogens led to an increase in hypophosphorylated pRb and to G_1 arrest. Decreases in expression of cyclin D1 preceded the antiestrogen-mediated cell cycle arrest. These changes were followed by a decrease in cdk2 kinase activity (52).

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More recently, direct regulation of cyclin D1 transcription by estrogen has been shown by others (2). In these recent studies, estrogen was able to overcome the cell cycle arrest imposed by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin by inducing cyclin D1 expression and pRb phosphorylation. The effects of estrogen were attributed to an estrogen-responsive regulatory region between −934 and −136 bp of the human cyclin D1 promoter. The activation of the cyclin D1 promoter by estrogen was independent of mitogen-activated protein kinase activity, which is inhibited by simvastatin (4).

To characterize more precisely the mechanisms by which estradiol induces cell cycle progression, we have studied MCF-7 cells arrested by tamoxifen. We present evidence that release of the cell cycle block by the addition of estrogen leads to rapid activation of cyclin E-cdk2 kinase and pRb phosphorylation. This occurs via a mechanism that is dependent upon induction of cyclin D1 by estrogen and a shift of the p21 cdk inhibitor from cyclin E-cdk2 to cyclin D1-cdk4 or cyclin D1-cdk6.

MATERIALS AND METHODS

Cell culture and synchronization. MCF-7 cells were obtained from M. Brown (Dana-Farber Cancer Institute, Boston, Mass.). They were routinely cultured in Dulbecco's modified essential medium (DMEM; Gibco-BRL) with 5% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.), penicillin (50,000 U/liter), streptomycin sulfate (50,000 μg/liter), and amphotericin B (Fungizone; 125 μg/liter). For tamoxifen synchronization, cells were plated between 5×10^3 and $10 \times 10^3/cm^2$. After 48 to 60 h, medium was changed to DMEM (phenol red free) with 5% charcoal-stripped serum (CSS) (HyClone, Logan, Utah), antibiotics, and 1 μM tamoxifen for 48 h (10). Cells were released from the arrest by addition of 500 nM 17β-estradiol or by changing the medium to fresh DMEM (phenol red free) with 5% CSS and 5 nM 17β-estradiol. For controls, cells were either given ethanol or changed to similar medium without estradiol. In some cases, the latter control contained 1 μM tamoxifen.

Cell cycle analysis. MCF-7 cells were plated for thymidine analysis on either 6-, 12-, or 24-well plates. Thymidine incorporation was assessed by labelling synchronized MCF-7 cells with 1 μCi of [*methyl*-^{3}H]thymidine per ml for 30 min. At the indicated time points, cells were washed once with phosphate-buffered saline (PBS) and once with 5% ice-cold trichloroacetic acid. Then, they were incubated in 5% trichloroacetic acid for at least 30 min on ice. After this incubation, cells were washed three times with water and lysed with 0.1 N NaOH. An aliquot of each sample was quantified by liquid scintillation counting.

For fluorescence-activated cell sorter (FACS) analysis, MCF-7 cells were harvested by trypsinization, pelleted gently, and resuspended in 2 ml of PBS. Cells were fixed by the gradual addition of 5 ml of 95% ethanol while being vortexed. After 30 min at room temperature, cells were stored at 4°C. Before processing, cells were collected by centrifugation and stained by addition of 1 ml of a 50-μg/ml propidium iodide solution. RNase A was added to these samples at a final concentration of 100 μg/ml, and the samples were incubated at room temperature for 15 min. Cell cycle analysis was carried out with a Becton Dickinson FACScan flow cytometer.

Preparation of cell extracts. Cell pellets were lysed for 20 min on ice in Nonidet P-40 (NP-40) lysis buffer (PBS [pH 7.2] containing in addition 250 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol [DTT], 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 50 μg of phenylmethylsulfonyl fluoride per ml, 1 mM NaF, 1 mM orthovanadate, 60 mM β-glycerophosphate). Cell debris was pelleted by centrifugation at 14,000 rpm for 15 min on an Eppendorf centrifuge (Brinkmann) at 4°C. The supernatant was assayed for protein content by Bradford analysis (Bio-Rad) and either used immediately or flash-frozen on dry ice and stored at −70°C.

Antibodies. Monoclonal antibodies against cyclin E and polyclonal antibodies against cdk2, cdk4, cdk6, p21, and p27 with their competing peptides when available were obtained from Santa Cruz Biotechnology, Santa Cruz, Calif. Monoclonal anti-pRb and polyclonal anti-cdk4 were purchased from Pharmingen, San Diego, Calif. Mouse monoclonal antibody against p27 was from Transduction Laboratories, Lexington, Ky. Monoclonal antibodies against p21 (CP-68 and CP-36) were a kind gift from B. Dynlacht, Harvard University, Cambridge, Mass. Monoclonal antibodies against human cyclin D1 and cyclin E were kindly provided by E. Harlow, Massachusetts General Hospital, Charlestown. Polyclonal antibody against p57 was a generous gift from S. Elledge, Baylor College of Medicine, Houston, Tex.

Western blot analysis and immunoprecipitation. Equal amounts of protein were processed for Western blot analysis by either sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel electrophoresis (PAGE) or SDS–12% PAGE. In general, 100 μg of protein per lane was separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore) by standard protocols. The membrane was blocked in PBS with 5% nonfat dry milk for 1 h. Subsequently, it was incubated for 3 h with a dilution of the specific antibody in PBS (2.5% nonfat dry milk, 0.05% Tween). After five washes with PBS (0.1% Tween), the filter was incubated for 1 h with a 1:5,000 dilution of horseradish peroxidase-linked secondary antibody (Jackson Laboratories). Immunodetection was achieved with an enhanced chemiluminescence system (Amersham).

For immunoprecipitations followed by Western blotting, lysates were incubated with the desired antibody cross-linked to beads for 3 h at 4°C with rocking. Beads were pelleted briefly on a microcentrifuge and washed twice with 1 ml of lysis buffer before electrophoresis and transfer to membrane. For immunodepletion, three sequential immunoprecipitations were carried out with each sample. An aliquot equivalent to 100 μg was taken from each supernatant for Western blot analysis to assess remaining proteins. Quantification of blots was done with PDI software (Huntington Station, N.Y.). Images were processed with Adobe Photoshop software and a Lacie Silverscanner II.

Kinase assays. For histone H1 phosphorylation, the amount of lysate immunoprecipitated varied with the specific antibody used in order to ensure that the kinase assay was conducted within the linear range. After immunoprecipitation, the beads were washed twice with lysis buffer and once with kinase assay buffer (20 mM Tris [pH 7.5], 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM DTT). Beads were incubated with 15 μl of kinase mix (kinase buffer containing 10 μM ATP with 2.5 μg of histone H1 [Boehringer Mannheim] and 16 μCi of [γ-^{32}P]ATP per reaction mixture) for 30 min at 30°C and stopped by addition of Laemmli sample buffer. Gels were stained with Coomassie blue, and excised bands were quantified by Cerenkov counting. To determine inhibitory activity present in extracts, equal amounts of protein or amounts as indicated were mixed and incubated at 30°C for 30 min before immunoprecipitation and kinase assay.

For glutathione *S*-transferase (GST)–Rb phosphorylation, we followed the conditions used previously to evaluate cdk4 activity from MCF-7 cells (15). In brief, cell pellets were lysed in Tween buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 10 μg of aprotinin per ml, 5 μg of leupeptin per ml, 50 μg of phenylmethylsulfonyl fluoride per ml, 5 mM NaF, 100 μM orthovanadate) for 30 min followed by centrifugation for 15 min at 4°C. A total of 500 μg of each extract was immunoprecipitated with cdk4 polyclonal antibodies (Pharmingen or Santa Cruz). Beads were washed twice with Tween buffer and three times with Rb-kinase buffer (50 mM HEPES [pH 7.5], 5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, 100 μM orthovanadate). The reaction was started by addition of 15 μl of Rb-kinase mix (Rb-kinase buffer with 30 μM ATP, 1 μg of GST-Rb [Santa Cruz], and 16 μCi of [γ-^{32}P]ATP per reaction mixture). After 30 min at 30°C, samples were mixed with Laemmli buffer and analyzed by SDS-PAGE.

cdc25A assay. For cdc25A assays, cyclin E-cdk2 complexes were immunoprecipitated with cyclin E antibodies. The immunoprecipitates were washed with cdc25A wash buffer (50 mM Tris [pH 7.9], 5 mM $MgCl_2$, 1 mM DTT) before addition of the reaction mix. The cdc25A reaction mix (50 mM Tris [pH 7.9], 5 mM $MgCl_2$, 10 mM DTT) contained when indicated recombinant GST-cdc25A (generous gift of Michele Pagano, New York University Medical Center, New York, N.Y.) alone or in the presence of 5 mM Na_3VO_4. The cdc25A reaction was carried out at 30°C for 30 min. The reaction was stopped by addition of 1 ml of cold NP-40 lysis buffer and processed for histone H1 kinase assays.

RESULTS

Characterization of estradiol-induced cell cycle reentry. We have used the estrogen-responsive MCF-7 human breast cancer cell line to study the effects of estradiol on the cell cycle. Exponentially growing cultures of MCF-7 cells were arrested in G_0/G_1 by treatment with 1 μM tamoxifen for 48 h in the presence of CSS. Synchronous release of the tamoxifen-arrested cells occurred after removal of tamoxifen and addition of fresh medium containing CSS and 17β-estradiol (5 nM estradiol). The results of a representative experiment are shown in Fig. 1A. Entry into S phase was determined by measuring thymidine incorporation. The peak of DNA synthesis was observed after 22 h of estradiol treatment, at which time the rate of thymidine incorporation increased more than 10-fold over uninduced levels. Tamoxifen-arrested MCF-7 cells that received only fresh medium containing 5% CSS or with 1 μM tamoxifen were not able to enter into S phase. FACS analysis of MCF-7 cells (Fig. 1B) confirmed that the tamoxifen-arrested cells ($T = 0$ h) were mostly in G_0/G_1 with a low percentage (8%) of cells in S phase. Following addition of fresh medium containing estradiol, over 50% of the cells had begun replicating their DNA by the time the peak of thymidine

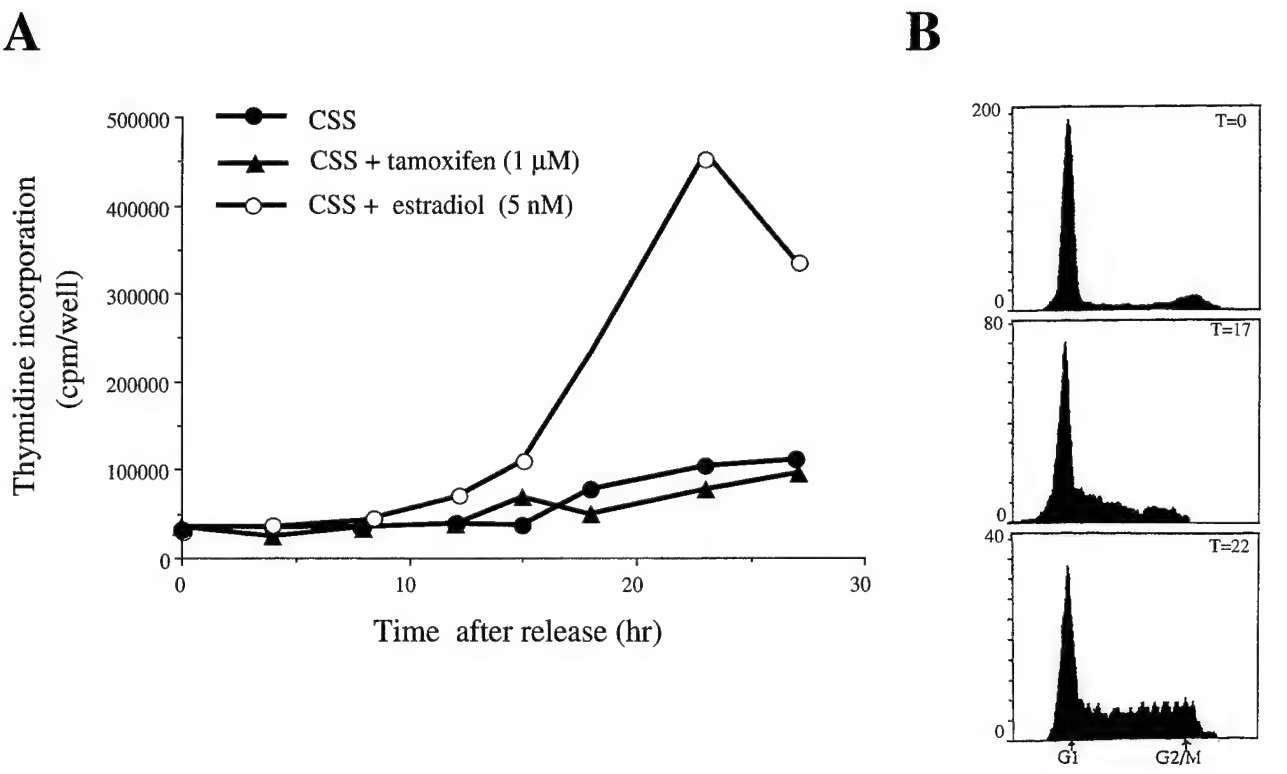

FIG. 1. Estrogen-dependent cell cycle progression. (A) MCF-7 cells were treated with tamoxifen for 48 h in the presence of 5% CSS. At $T = 0$ h, medium was changed to 5% CSS alone or with addition of either 1 μM tamoxifen or 5 nM estradiol. At the times indicated, cells were pulsed with [*methyl*-^{3}H]thymidine for 30 min and thymidine incorporation was determined as described in Materials and Methods. (B) MCF-7 cells treated for 48 h with tamoxifen (0 h) or after change to 5% CSS with 5 nM estradiol for the indicated times (in hours) were fixed, incubated with propidium iodide, and analyzed by FACS. The *y* axes show the number of cells.

incorporation occurred ($T = 22$ h). Synchronized cell cycle reentry of tamoxifen-blocked cells could also be induced by adding relatively high concentrations of estradiol (500 nM) to cells in the continued presence of the tamoxifen blocking agent (data not shown).

Phosphorylation of the Rb protein and escape from tamoxifen inhibition. Previous work addressing the action of tamoxifen on the cell cycle had indicated that MCF-7 cells are sensitive to the antiestrogen tamoxifen and other similarly acting compounds only in a narrow window of time in the cell cycle in early to mid-G_1 (35, 50). However, these studies did not evaluate the effect of tamoxifen on the cell cycle machinery. The period of responsiveness to tamoxifen in G_1 is reminiscent of the effects of transforming growth factor β (TGF-β) on cell cycle advance, which are known to involve primarily a blockage of pRb phosphorylation (14, 25). Thus, after cells have phosphorylated their complement of pRb in late G_1, they become nonresponsive to the growth-inhibitory effects of TGF-β.

For these reasons, we determined whether the acquired refractoriness to tamoxifen in late G_1 could be correlated with phosphorylation of pRb. To do so, we arrested cells with tamoxifen for 48 h and then released them by addition of fresh medium containing 5% CSS and 5 nM estradiol. At various time points thereafter, estradiol-containing medium was removed and replaced with tamoxifen-containing medium. Effects on cell cycle advance were ascertained by measuring the subsequent ability of these cells to incorporate thymidine at $T = 22$ h, the time of peak thymidine incorporation by control cells that had not been treated with tamoxifen following the estradiol-induced cell cycle progression.

As shown in Fig. 2A, most of the cells could be prevented from subsequent S-phase entry if tamoxifen was added back immediately after estradiol addition at $T = 0$ h. However, by 6 h, virtually all the cells were refractory to tamoxifen treatment, achieving levels of thymidine incorporation similar to those of cells that had been exposed continuously to estradiol for 22 h. These data indicated that, within several hours after estradiol addition, tamoxifen (and presumably the ER) no longer exerted control over cell cycle advance.

Western blot analysis of the Rb protein indicated that phosphorylation of pRb correlated closely with the acquisition of refractoriness to tamoxifen (Fig. 2B, top). Densitometric analysis of the different pRb forms revealed the almost complete disappearance of the hypophosphorylated form and its replacement by the hyperphosphorylated form by 6 h. Indeed, the ratio of hypo- to hyperphosphorylated forms dropped dramatically in the first few hours after estradiol stimulation (Fig. 2B, bottom). Surprisingly, the kinetics of pRb phosphorylation preceded by many hours the entry into S phase and differed in this way from the schedule of changes normally seen during cell cycle progression from G_0/G_1 to S phase (6, 9).

Such acquired resistance in mid- to late G_1 to growth inhibition has been observed in a number of other cases besides the aforementioned nonresponsiveness to TGF-β seen in late G_1. Thus, introduction of low levels of cycloheximide or removal of mitogens has been observed to block G_1 advance when applied early in this phase but not in the last several hours of this phase (29, 37, 38). In each case, the time of acquired resistance has been equated with passage through a restriction point (R point). By extension, the acquired refractoriness to tamoxifen inhibition also represents a restriction point transition. As with the other operationally defined R

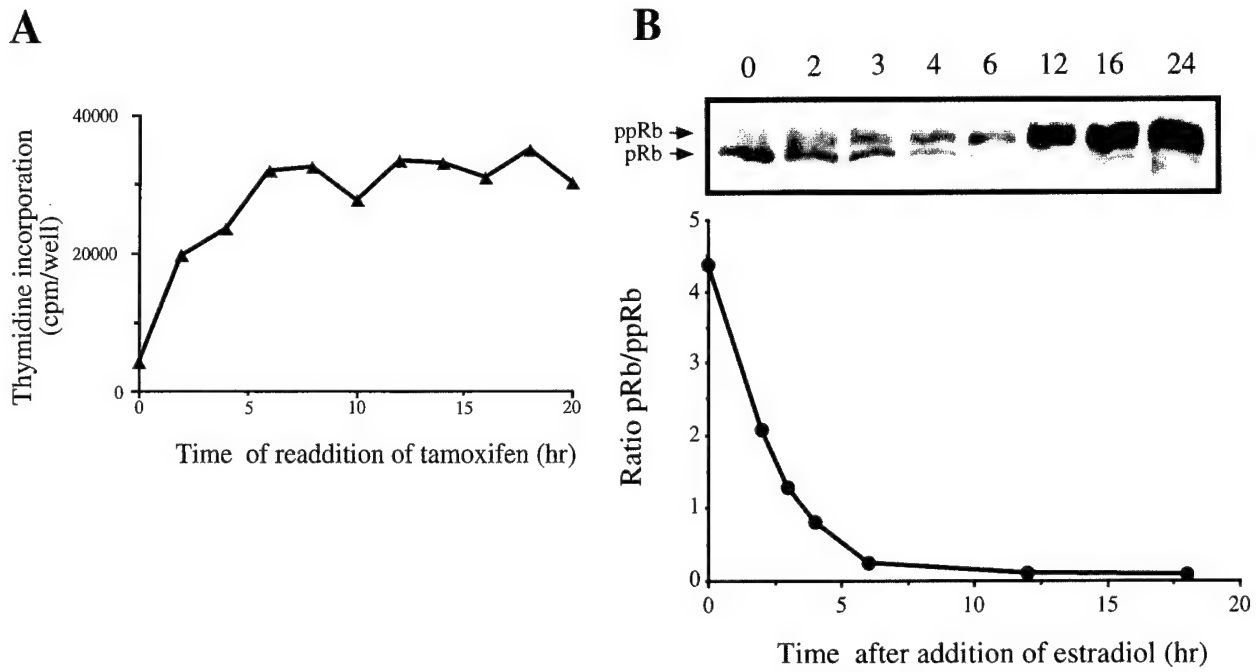

FIG. 2. Refractoriness to tamoxifen and phosphorylation of the retinoblastoma protein (pRb). (A) Tamoxifen-arrested MCF-7 cells were stimulated with 5 nM estradiol at $T = 0$ h. At the indicated times, the medium was replaced with 5% CSS containing 1 μM tamoxifen. After 22 h, all the cells were pulsed with [*methyl*-^{3}H]thymidine and processed as described in Materials and Methods. (B) (Top) Total cell extracts of MCF-7 cells (100 μg) at different time points after release (in hours) from tamoxifen-induced cell cycle block were analyzed for pRb protein by Western blotting. (Bottom) Densitometric analysis of pRb phosphorylation. The ratio of hypophosphorylated pRb to hyperphosphorylated pRb (ppRb) was plotted as a function of time after estradiol addition.

points, this transition is closely contemporaneous with and likely connected with the phosphorylation of pRb.

Regulation of cdks by estradiol in MCF-7 cells. Since pRb phosphorylation is known to be driven largely by cdks, we characterized the effects of estradiol on the activity of the cyclin-cdk complexes implicated in this phosphorylation in mid- to late G_1. Recent reports have suggested that the cyclin D1-cdk4 complex may be a direct target of estrogen action (see Introduction). For this reason, we first characterized the changes in cyclin D1 protein and cyclin D1-cdk4 kinase activity following estradiol addition to tamoxifen-arrested MCF-7 cells. To avoid the possible confounding effects of freshly added serum, we reversed the cell cycle block by adding only estradiol (500 nM final concentration) to tamoxifen-arrested cells. The control cells received only the solvent vehicle (ethanol).

The results shown in Fig. 3A (top) confirmed that estradiol can induce expression of cyclin D1 protein very rapidly. Thus, an increase of cyclin D1 levels was already apparent within 2 h of treatment. Levels of cyclin D1 continued to increase steadily, achieving fivefold higher levels by 6 h. Taken together with the previously reported work (2), the present results indicated a specific effect of estrogen on cyclin D1 levels.

In parallel with these measurements of cyclin D1 levels, we measured cdk4 activity by using GST-Rb as a substrate and antibodies against a peptide derived from the carboxy terminus of cdk4 to immunoprecipitate cyclin D1-cdk4 kinase complexes. We assumed here that the activity of cdk4 could be used as well as an index of the activity of the similarly regulated cdk6 enzyme. As a negative control, we blocked specific binding by preincubating the antibodies with the antigenic peptide. Figure 3A (bottom) shows the changes of cdk4-dependent kinase following estrogen release of tamoxifen-arrested cells. Considerable cdk4 activity toward the GST-Rb substrate was apparent in the tamoxifen-arrested cells prior to their release by estradiol compared to the negative control. After addition of estradiol, this activity increased slowly and steadily, starting at 2 h after addition of estradiol and peaking by 6 h, ultimately reaching threefold higher levels. Similar results were obtained when the kinase assay was performed after immunoprecipitation with cyclin D1-specific antibodies (reference 2 and data not shown). Indeed, we anticipated that we would observe similar kinetics of enzyme activation following immunoprecipitation with either anti-cdk4 or anti-cyclin D1 antibody. While the other D-type cyclins, D2 and D3, are equally able to activate cdk4, our work and that of others have shown that cyclin D2 is not detectable in these cells and cyclin D3 is present in low and constant amounts following estradiol treatment (reference 53 and data not shown).

In addition, we also investigated the status of the other important G_1 cyclin-cdk complex, cyclin E-cdk2. In this instance, cyclin E-cdk2 activity was assayed with immunoprecipitates obtained with an anti-human cyclin E monoclonal antibody and histone H1 as substrate. A dramatic induction of this kinase activity that was first apparent within 2 h of estradiol addition was observed (Fig. 3B, top). At 6 h, this activity had already peaked at a level that was ~20 times higher than that seen at $T = 0$ h (Fig. 3B, bottom). A similar relative induction was seen when cdk2-specific antibodies were used to prepare the precipitates analyzed in the kinase assay (see Fig. 6A). Analysis of cyclin A-dependent kinase activity in the same extracts showed a delayed induction of this enzyme (Fig. 3B, middle) with respect to that of cyclin E with maximum levels reached by 24 h (Fig. 3B, bottom). This induction correlated well with entrance of the cells into S phase.

The changes in cyclin D1-cdk4 kinase could be explained by the increase in cyclin D1 expression. Possible changes in the levels of INK4A were not considered as MCF-7 cells carry a

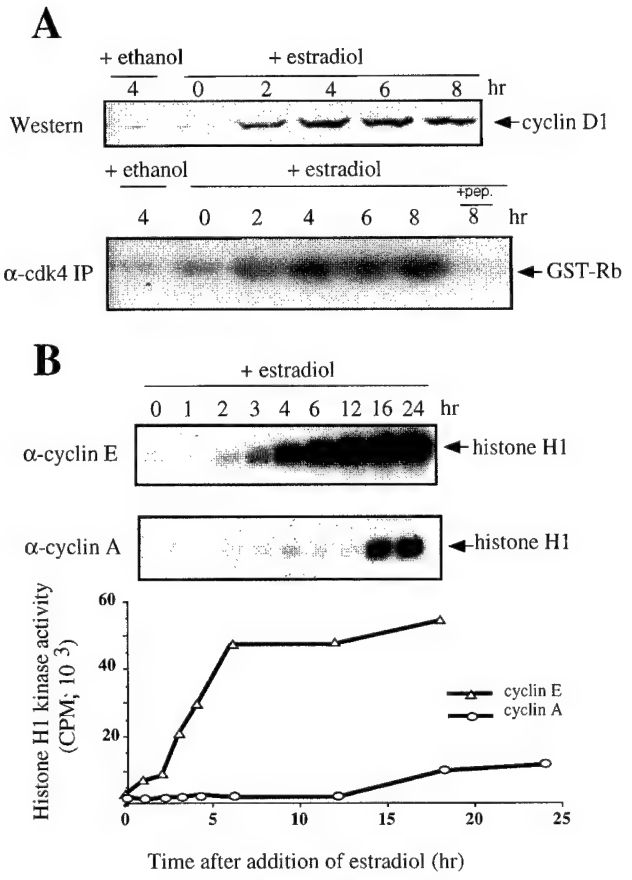

FIG. 3. Effect of estradiol on G_1 cdks. (A) Changes in cyclin D1-cdk4 after estradiol addition. (Top) Western blot analysis of cyclin D1 expression at the indicated time points after the addition of 500 nM estradiol or ethanol to tamoxifen-arrested cells. (Bottom) The same extracts were immunoprecipitated with a polyclonal antibody against the carboxy terminus of cdk4. The kinase activity of these immunocomplexes was measured with GST-Rb as substrate. Background activity (lane +pep.) was determined by blocking cdk4 antibody with a specific antigenic peptide. (B) (Top and middle) Activity of cyclin E (top)- or cyclin A (middle)-associated kinases was assessed with histone H1 as substrate. Tamoxifen-arrested MCF-7 cells were released by a change to 5% CSS with 5 nM estradiol. Cells were harvested and processed for histone H1 kinase assays at the times indicated as described in Materials and Methods. (Bottom) Quantitation of kinase activities. The amount of ^{32}P incorporated into histone H1 in counts per minute was obtained by determining the Cerenkov counts on the excised histone H1 bands.

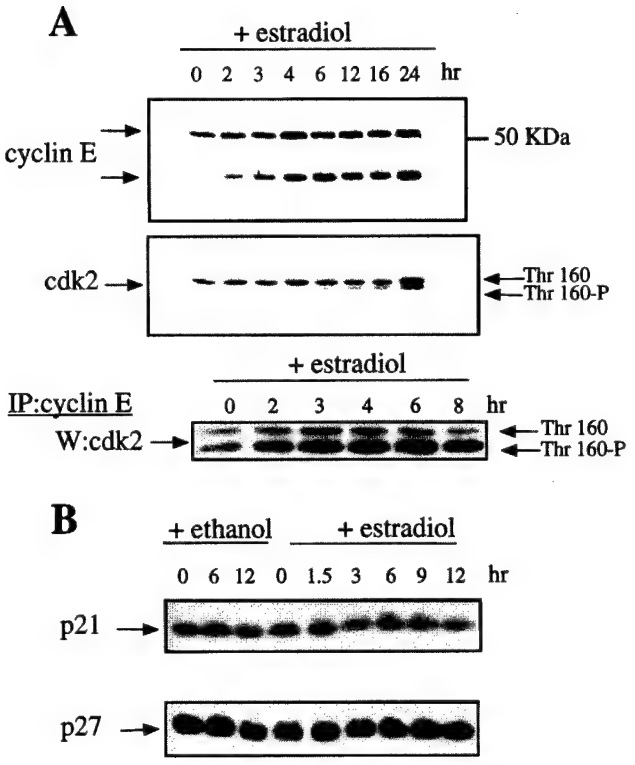

FIG. 4. Effect of estradiol on components of the cyclin E-cdk2 complex. (A) Analysis of cyclin E (top), cdk2 (middle), and cyclin E-associated cdk2 (bottom) from extracts obtained at different time points after release from the tamoxifen block by change to 5% CSS–5 nM estradiol. W, Western blot antibody; IP, immunoprecipitating antibody. (B) Western blot analysis of cell cycle inhibitors, p21 (top) and p27 (bottom), from tamoxifen-arrested cells that received either ethanol or estradiol.

deletion of this gene (31). However, the induction of cyclin E-cdk2 kinase activity still required explanation. Thus, we initiated further experiments to determine how cyclin E-dependent kinase activity was being inhibited by tamoxifen treatment and induced by subsequent estradiol treatment.

Expression of cyclin E, cdk2, and cyclin E-cdk2 inhibitors following estradiol release of tamoxifen-arrested MCF-7 cells. The most likely explanation for the observed changes in the activity of the cyclin E-cdk2 complexes was that estrogen, acting through its receptor, modulated the levels of cyclin E, cdk2, or associated regulatory molecules. Indeed, addition of actinomycin or cycloheximide at the time of estradiol treatment prevented cyclin E-cdk2 activation by estradiol, indicating the need for protein synthesis following estradiol addition (data not shown). However, this need for de novo protein synthesis could not be explained by a requirement for increased levels of cyclin E, since Western blot analysis indicated that the levels of a major form of cyclin E were relatively constant in the 6 h following estradiol addition (Fig. 4A, top). Occasionally, increases in a more rapidly migrating form of cyclin E (22, 23) were apparent during this time period; these increases were not observed reproducibly and could not be correlated with increases in cyclin E-associated kinase activity. The levels of the cdk2 protein also remained essentially unchanged until 24 h (Fig. 4A, middle), long after the functional activation of the cyclin E-cdk2 complexes. A slight increase in the phosphorylated form of cdk2 was seen at 6 h. However, a more remarkable increase in this active form was seen only much later, coinciding with the greatest accumulation of cells in S phase (Fig. 1).

It was also possible that estradiol affected the assembly of cyclin E-cdk2 complexes. To assess this possibility, we analyzed the changes in the levels of the cyclin E-associated cdk2 following estradiol addition to tamoxifen-arrested cells. Cell lysates prepared at different times after estradiol addition were immunoprecipitated with cyclin E-specific antibodies followed by Western blot analysis using anti-cdk2 antibody as probe. This analysis revealed that cyclin E-cdk2 complexes were already present during the tamoxifen arrest and that their levels did not change substantially following estradiol treatment (Fig. 4A, bottom). There was a small increase in the phosphorylated, active cdk2 form following estradiol addition. However, no changes were seen in the cdk-activating kinase activity following treatment with estradiol (data not shown). Together, these data indicated that changes in cyclin E and cdk2 levels or their association with one another could not explain the marked increases in the activity of the cyclin E-cdk2 complexes. This

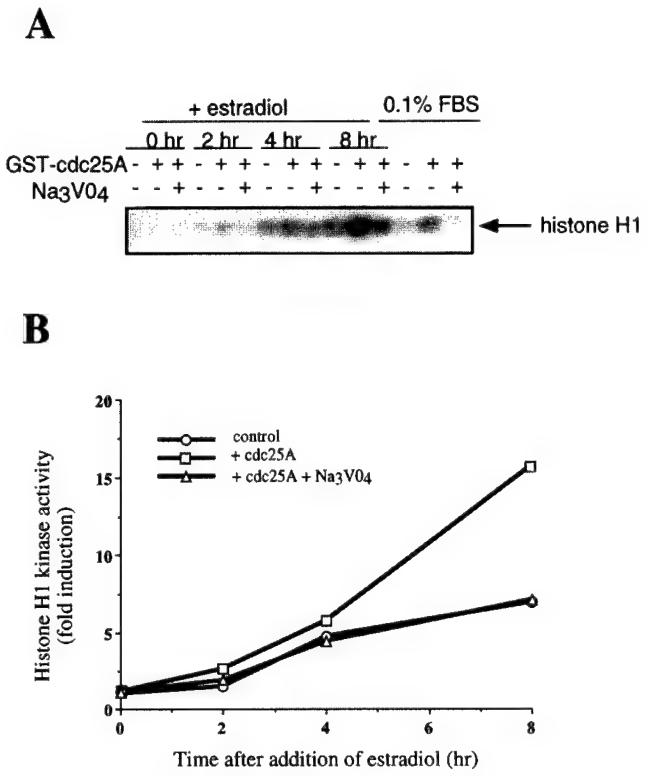

FIG. 5. Effect of recombinant cdc25A on cyclin E-cdk2 activity. (A) Asynchronous MCF-7 cells were treated with either 5% CSS with 1 μM tamoxifen or 0.1% fetal bovine serum (FBS) for 48 h. Tamoxifen-arrested cells were then treated with estradiol for the times shown. Cyclin E-cdk2 complexes were immunoprecipitated from the indicated cell extracts with antibodies specific for cyclin E. The immunocomplexes obtained were split into three and incubated in cdc25A reaction mix in either the presence or the absence of recombinant GST-cdc25A and sodium orthovanadate. (B) Graphic representation of cyclin E-associated histone H1 kinase activity obtained from results shown in panel A.

suggested in turn that other regulators of cyclin-cdk activity were responsible for modulating cyclin E-associated kinase activity following estradiol treatment. More specifically, cdk inhibitors were attractive candidates for such a role. Hence, we determined whether expression of any of the known cdk2 inhibitors was affected by estrogen.

As shown in Fig. 4B, levels of the p21 (top) or p27 (bottom) cdk inhibitors did not change substantially during the hours preceding cyclin E-cdk2 activation. The p57 cdk inhibitor was not detectable in these cells (data not shown). Levels of the INK4 class of inhibitors were not monitored here, as these inhibitors affect only cdk4 and cdk6. Therefore, we concluded that the observed changes in cyclin E-cdk2 activity following estradiol treatment could not be the result of changes in the overall levels of the known cdk2 inhibitors.

Role of cdc25A in cyclin E-cdk2 activation. Cyclin E-cdk2 complexes can also be activated by dephosphorylation mediated by the cdc25A phosphatase (30). Thus, it was possible that the cyclin E-cdk2 complexes from tamoxifen-arrested cells were held inactive by the inhibitory phosphorylation. To address this possibility, we tested the ability of recombinant GST-cdc25A to activate cyclin E-cdk2 complexes immunoprecipitated from cell extracts prepared at different times after estradiol addition. The results shown in Fig. 5A indicated that cdc25A was not able to activate the complexes obtained from tamoxifen-arrested cells. Nevertheless, there was a gradual increase in the ability of cyclin E-cdk2 complexes to be activated by cdc25A in complexes obtained from cells treated with estradiol. This activation was abolished in the presence of the tyrosine phosphatase inhibitor Na_3VO_4 (Fig. 5A). Quantitation of the activation induced by cdc25A indicated that, while cyclin E-cdk2 complexes obtained from tamoxifen-arrested cells were not significantly activated, complexes from cells treated with estradiol for 8 h were activated more than twofold (Fig. 5B). A similar fold activation was also seen when cyclin E-cdk2 complexes obtained from serum-starved MCF-7 cells were used (Fig. 5A, last three lanes). Taken together, these results suggested that the absence of cyclin E-cdk2 kinase activity during tamoxifen-mediated cell cycle arrest was not due to inhibitory phosphorylation of cdk2 attributable in turn to the lack of cdc25A activity.

Analysis of cdk-inhibitory activity in tamoxifen-arrested cells. The absence of substantial changes in the levels or functioning of the various molecules that contribute to cdk2 activity caused us to undertake direct biochemical analysis of cdk2 complexes prepared from cells treated with tamoxifen or estradiol. In particular, prior to immunoprecipitation we mixed extracts from tamoxifen-blocked cells (0 h) with those prepared from cells that had been released from the tamoxifen block by 8 h of estradiol treatment (8 h). In doing this, we hoped to determine whether the tamoxifen-blocked cells contained a soluble inhibitor of cdk2 activity.

The results of these in vitro assays, shown in Fig. 6A, indicated that tamoxifen-arrested cells did indeed contain an activity capable of reducing the activity of cdk2 to levels as low as those observed with extracts from tamoxifen-arrested cells. This inhibition of estradiol-treated extracts was seen when either cyclin E- or cdk2-specific antibodies were used to immunoprecipitate cyclin E-cdk2 complexes prior to assay for kinase activity (Fig. 6A). Moreover, this inhibition was seen only when the extract mixtures were preincubated at 30°C, not when they were preincubated at 4°C (Fig. 6A, last two lanes).

These findings pointed to the presence of a soluble inhibitory substance in tamoxifen-arrested cells capable of abolishing the activity of cyclin E-cdk2 complexes. We assumed tentatively that this inhibitory substance detected in vitro was responsible for the observed inhibition of cyclin E-cdk2 activity in tamoxifen-arrested cells.

Changes in inhibitory activity after estradiol-induced release of the cell cycle block. We wished to monitor the fate of this inhibitory substance following the release of cells from the tamoxifen block. To do so, we tested the inhibitory activity from cells prepared at different time points after estradiol addition. In parallel, we also measured the activity of cdk2-dependent kinase from the same extracts. Figure 6B (left) shows high levels of cyclin E-cdk2 activity at 8 h after addition of estradiol. Mixing of this extract (T = 8 h) with an extract prepared from tamoxifen-arrested cells led to complete inactivation of the induced activity (Fig. 6B, right) in confirmation of the results reported above. The inhibitory activities obtained by mixing cell extracts prepared from cells at various times after estradiol treatment were then compared with the inhibitory activity observed in this 0-h extract (100%). Extracts prepared from cells that had been treated for 2 h with estradiol had only 40% of the inhibitory activity of the tamoxifen-arrested extract, and this activity was reduced further at 4 h, being undetectable by 8 h of estradiol treatment.

These results indicated that estradiol treatment caused the rapid loss of the inhibitory activity that had accumulated during the tamoxifen-imposed cell cycle block. However, the fact that the disappearance of the inhibitory activity did not lead immediately to a reciprocal increase in cdk2 kinase activity suggested that other cdk regulators such as the cdk-activating

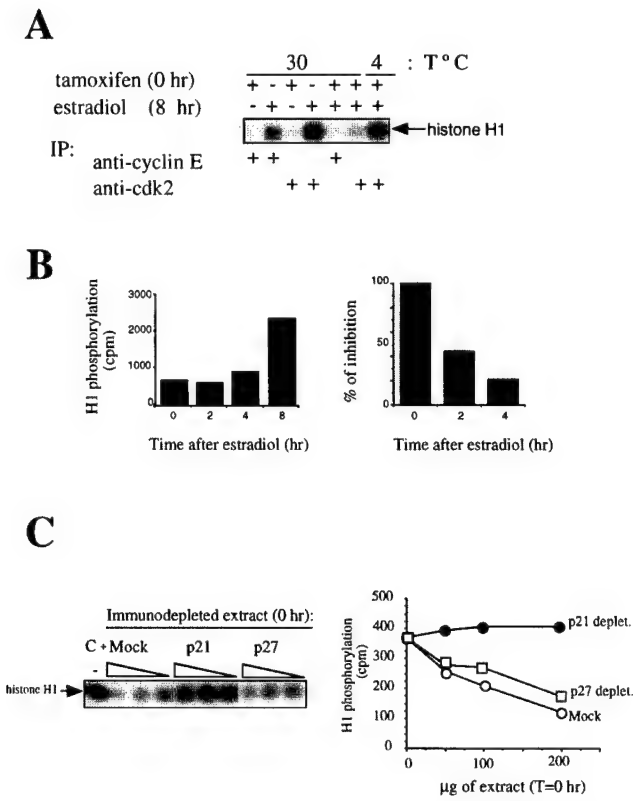

FIG. 6. Characterization of cdk2-inhibitory activity present in tamoxifen-arrested cells. (A) Temperature-dependent inhibition of cyclin E- or cdk2-associated kinase activity. Extracts from tamoxifen-arrested cells and estradiol-stimulated cells alone or mixed as indicated were subjected to immunoprecipitation with cyclin E- or cdk2-specific antibodies. The immunocomplexes were then assayed for kinase activity against histone H1. (B) Changes in cdk2-dependent kinase activity (left) and inhibitory activity (right) after addition of 0.5 μM estradiol to tamoxifen-arrested cells. (C) Immunodepletion of inhibitory activity against cyclin E-cdk2 kinase. (Left) Extracts from tamoxifen-arrested cells were immunodepleted with antibodies against p21 (CP-68) and p27 (SC-528) or irrelevant antibodies (mock). The inhibitory activity remaining after immunodepletion was evaluated by mixing the respective supernatants with extracts from estradiol-treated samples (8 h) as for panel A. The mixture was incubated for 30 min at 30°C followed by a standard immunoprecipitation and kinase assay against histone H1. The use of other antibodies against either cdk inhibitor gave similar results. (Right) Quantitation of the results from immunodepletion (left). The level of histone H1 phosphorylation was plotted against the amount of extract from each immunodepletion that was mixed with 100 μg of extracts from cells at 8 h poststimulation.

kinase may be required to achieve full activation of cyclin E-cdk2.

Depletion of cdk2 inhibitors in tamoxifen-arrested cells. Obvious candidates for the cyclin E-cdk2 inhibitory activity described above were the cdk inhibitors, specifically, p21 and p27. While their overall levels in the cell did not change following estradiol treatment, it was possible that they underwent relocalization in the cell in response to estradiol. Accordingly, we attempted to associate the observed inhibitory activity with specific cdk inhibitors, doing so by treating the inhibitory extract with antibodies reactive with one or another of these proteins in order to deplete these molecules. Following antibody treatment, we tested the remaining supernatants for any inhibitory activity that survived immunodepletion by mixing them with the 8-h extract.

We immunodepleted either p21 or p27 from the tamoxifen-treated (0 h) extract with anti-p21 or anti-p27 specific antibodies, respectively. Immunodepletion was performed by three sequential immunoprecipitations to ensure greater than 99% removal of the inhibitor (see Fig. 7B or Fig. 8). Different amounts of extract (T = 0 h) were used to quantify more precisely any resulting changes in inhibitory activity. As shown in Fig. 6C (left), immunodepletion with anti-p21 antibody efficiently abolished the inhibitory activity of the extract prepared from tamoxifen-arrested cells compared to mock-depleted extracts. In contrast, immunodepletion of p27 led to only a slight reduction of the inhibitory activity of the tamoxifen-treated cell extracts (Fig. 6C, right). These observations suggested that the great bulk of the cyclin E-cdk2 inhibitory activity present in the tamoxifen-treated cell extracts was due to p21 molecules present in these extracts while a minor component was due to p27.

Other evidence indicated that p27 was indeed present in these extracts although not in a configuration that permitted it to inhibit cyclin E-cdk2 activity under our mixing conditions. Thus, when extracts from tamoxifen-treated cells were heated to 100°C prior to assay of cdk2-inhibitory activity, greater than 60% of the inhibitory activity present was due to p27 as judged by immunodepletion with anti-p27 antibodies (data not shown). This also indicated that in tamoxifen-arrested MCF-7 cells the levels of p27 as determined by this functional assay were actually greater than those of p21. Hence, in tamoxifen-treated cell extracts, the great bulk, and perhaps all, of the available, active cdk2-inhibitory substance was derived from p21 molecules. p27 molecules, though present in the extracts, were sequestered in heat-labile complexes from which they could be liberated by brief heating. Moreover, as shown in Fig. 6B, this soluble p21-associated inhibitory activity declined dramatically during the hours following estradiol treatment.

Characterization of complexes between p21 and G_1 cyclin-cdks. As shown above (Fig. 3A), estrogen treatment of the MCF-7 cells causes them to express increased levels of cyclin D1. The resulting cyclin D1-cdk4 complexes might act to bind increasing proportions of the cell's pool of p21 and p27 molecules, thereby abstracting them from cyclin E-cdk2. Indeed, just such a model of cdk inhibitor action has also been proposed to operate in other cell types (46). With this model in mind, we determined the changes in the association of p21 with cyclin D1-cdk4 and cyclin E-cdk2 following estradiol addition with the same extracts analyzed previously to test cdc25A activation.

As expected from the results of earlier experiments, levels of cyclin D1 increased very rapidly, reaching levels fourfold higher than those of control by 6 h (Fig. 7A, top). Moreover, immunoprecipitation of p21 followed by Western blot analysis for cyclin D1 revealed an increase in p21-associated cyclin D1 after addition of estradiol (Fig. 7A, bottom). This increase was first seen within 2 h of estradiol treatment and peaked by 6 h with fourfold higher levels than those that were seen in tamoxifen-blocked cells. Therefore, these results indicated that increases in total levels of cyclin D1 by estradiol correlated in time and magnitude with the changes in levels of p21 associated with cyclin D1. The activation of cyclin E-cdk2 complexes occurred at approximately the same time as these changes, suggesting a possible link between the events (Fig. 5).

The observed increases in p21 associated with cyclin D1 following estradiol treatment were compatible with a model in which D1-cdk4 complexes compete with cyclin E-cdk2 complexes for a limited pool of p21 and abstract increasing amounts of p21 from cyclin E-cdk2 complexes following estradiol treatment. However, the above data did not prove this point, as they did not reveal the proportion of total cellular p21 that was present in these two types of cyclin-cdk complexes at various times after estradiol treatment.

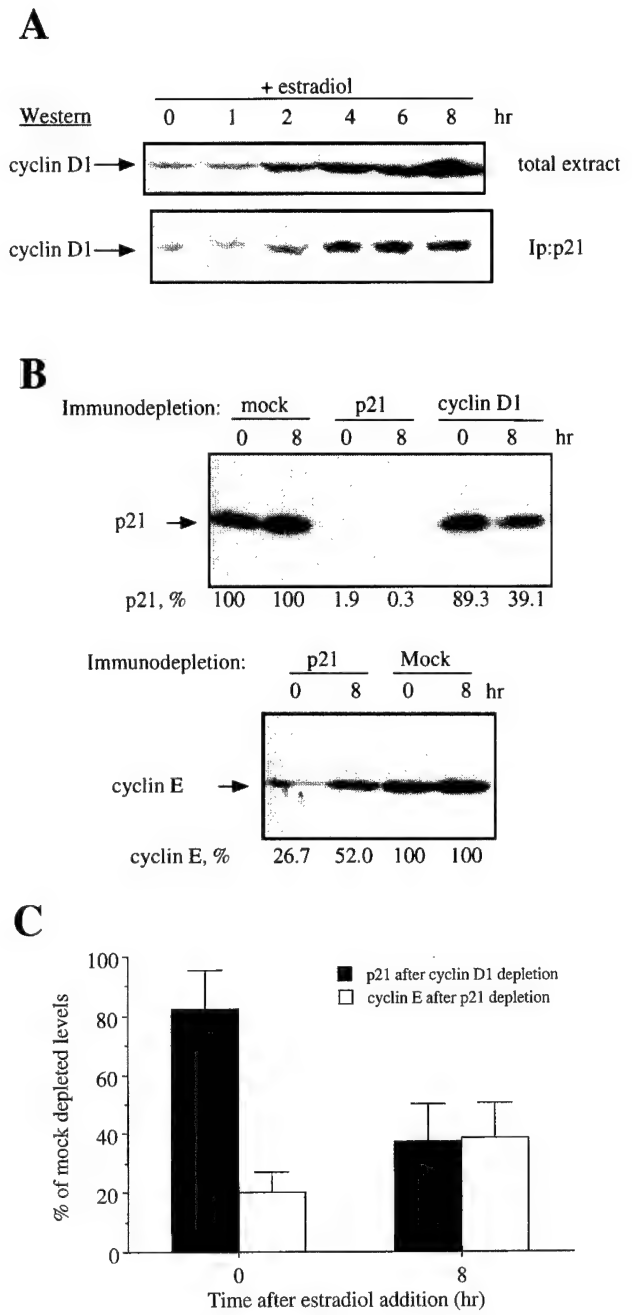

FIG. 7. Changes in p21 distribution among G_1 cyclin-cdk complexes (A). (Top) Cyclin D1 expression from MCF-7 cells after estradiol addition was determined by Western blot analysis of total cell extracts at the time points indicated. (Bottom) Levels of cyclin D1 associated with p21. Cell extracts obtained from tamoxifen-arrested cells or at different times after stimulation with estradiol were subjected to immunoprecipitation with p21 antibodies. The immunoprecipitated complexes were then analyzed for the presence of cyclin D1 by Western blot analysis. (B) (Top) Cell extracts from $T = 0$ and $T = 8$ h after addition of estradiol were immunodepleted by three sequential immunodepletions with nonrelevant antibodies (mock) or with antibodies against either p21 (SC-397) or cyclin D1 (HD-33). The amount of p21 surviving immunodepletion was evaluated by Western blot analysis of 100 μg of total protein with p21-specific antibodies. Numbers at the bottom of each lane represent the amount of specific protein remaining in the supernatant after immunodepletion as determined by densitometric quantitation of images. (Bottom) Levels of cyclin E surviving mock or p21 immunodepletion. The amount of cyclin E remaining in supernatants from mock-depleted extracts was taken as 100%. (C) Quantitation of three independent experiments similar to those described for panel B in which levels of p21 and cyclin E were monitored simultaneously after cyclin D1 and p21 immunodepletion, respectively. Error bars indicate the standard deviations of the samples.

To approach this question directly, we undertook immunodepletion studies with cyclin D1- and p21-specific antibodies. The immunodepleted extracts were then examined for the levels of p21 remaining by Western blot analysis (Fig. 7B, top). Quantitation of p21 levels indicated that p21 immunodepletion removed over 99% of p21 levels from extracts obtained at $T = 0$ h or $T = 8$ h. Anti-cyclin D1 antibodies were similarly successful in removing cyclin D1 from cell extracts (data not shown). When anti-cyclin D1 antibodies were used for immunodepletion, about 10% of the cells' complement of p21 could be removed from extracts prepared at $T = 0$ h while more than 60% of the p21 pool could be immunodepleted from estradiol-treated extracts ($T = 8$ h). Therefore, within 8 h of estradiol treatment, the bulk of cellular p21 associated with cyclin D1-cdk4. We concluded that much and perhaps all of the observed decrease in cdk2-inhibitory activity observed in cell extracts from estradiol-treated cells could be ascribed to this redistribution of p21 to cyclin D1-cdk4 complexes.

To address whether the increase in association of p21 with cyclin D1 coincided with a decrease in the amount of cyclin E complexed with p21, we performed the following experiment. Cell extracts from tamoxifen-arrested cells or from cells treated with estradiol for 8 h were subjected to immunodepletion with p21 antibodies. As shown in Fig. 7B (bottom), the percentage of cyclin E that was not bound by p21 increased from 27% ($T = 0$ h) to 52% after 8 h of estradiol treatment. These results indicated that the activation of cyclin E-cdk2 complexes (Fig. 3B and Fig. 5) correlated with the increase of the amount of cyclin E not complexed to p21. To further strengthen the correlation between the increase in cyclin D1 binding to p21 and the release of p21 from cyclin E-cdk2, we repeated the immunodepletion experiment with several independently obtained samples. The results of three of these experiments are shown in Fig. 7C. The levels of p21 associated with cyclin D1 increased on average from ~20 to ~60% while the levels of cyclin E associated with p21 decreased from ~80 to ~60% following estradiol treatment.

The experiments described above measured the exchange of p21 between cyclin E-cdk2 and cyclin D1 after 8 h of estradiol treatment. However, the induction of cyclin D1 protein levels already occurred between 2 and 4 h after estradiol addition (Fig. 3A and 7A). In order to determine the amount of cyclin E associated with p21 during the period when cyclin D1 expression was induced, we performed an immunodepletion with anti-p21 antibodies at different times poststimulation with estradiol, measuring the amounts of residual cyclin E in cell lysates that survived immunodepletion and were therefore not complexed with p21. An increase in the levels of cyclin E that was not associated in complexes with p21 was evident in lysates prepared from cells at 2 h after estradiol treatment and reached a maximum in lysates prepared after 6 h of estradiol treatment (data not shown). These results correlated with the increase in p21 association with cyclin D1 as shown in Fig. 7A (bottom) and, more importantly, with the increase in cyclin E-cdk2-associated kinase activity (Fig. 5). Taken together, these results indicated that the activation of the cyclin E-cdk2 complex after estradiol stimulation is the result of the redistribution of p21 from cyclin E to cyclin D1.

Characterization of active cyclin E-cdk2 complexes. The data described above suggested a strong correlation between increases in cyclin E-cdk2 complexes devoid of p21 and increases in cyclin E-cdk2 activity. This correlation argued that the pool of cyclin E-cdk2 free of p21 was responsible for the cyclin E-associated histone H1 kinase. However, cyclin E-cdk2 complexes can also be regulated by bound p27 molecules, making it possible that p27 was binding and regulating cyclin

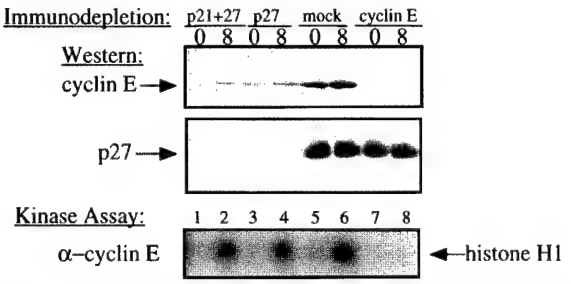

FIG. 8. Characterization of the presence of p27 in cyclin E-cdk2 complexes and formation of active cyclin E-cdk2 complex. Cell extracts from tamoxifen-arrested cells and at 8 h after release by estradiol (times are indicated in hours at the top of the figure) were immunodepleted with nonspecific antibodies (mock) or the indicated specific antibodies. For double immunodepletion of p21 and p27 (p21+p27), a mixture of both specific antibodies was used in the three sequential immunodepletions. (Top) Western blot analysis of cyclin E remaining in the supernatant after immunodepletion. A total of 100 μg of total protein from each supernatant was analyzed. (Middle) Western blot analysis of p27 remaining in the supernatant after immunodepletion. (Bottom) Cyclin E-dependent kinase activity against histone H1 was assayed in supernatants after respective immunodepletions.

E-cdk2 activity in a manner similar to that of p21. For this reason, we determined if the association between p27 and cyclin E-cdk2 complexes was affected by estradiol. Immunodepletions with anti-p27 antibodies were performed on extracts obtained from cells either arrested by tamoxifen or released by estradiol for 8 h with anti-p27 antibodies. p27 antibodies were able to immunodeplete similar levels of cyclin E (>80%) at both time points (Fig. 8, top; compare lanes 3 and 4). In this experiment, p27 was efficiently immunodepleted by the specific antibodies where no effect on p27 was observed in mock-depleted extracts (Fig. 8, middle). To our surprise, either the anti-p21 or the anti-p27 antibody could deplete the majority (over 60%) of cyclin E. One explanation for this apparent discrepancy is that some cyclin E-cdk2 complexes bind p21 and p27 molecules simultaneously. Nonetheless, these results did indicate that changes in p27 association with cyclin E-cdk2 could not explain the observed increases in cyclin E-cdk2 activity following estradiol treatment.

These conclusions were based on the notion that association of cyclin E-cdk2 complexes with either p21 or p27 resulted in the functional inactivation of the cyclin-cdk complex. To validate this directly, we immunodepleted lysates with p21 and p27 antibodies simultaneously. An initial experiment indicated that such immunodepletion was able to completely remove cyclin E (~99%) from cells arrested with tamoxifen (Fig. 8, top, lane 1). The same immunodepletion of p21 and p27 from extracts of estradiol-treated cells containing active cyclin E-cdk2 complexes still immunodepleted the bulk (90%) of cyclin E (lane 2). These results indicated that, even at the peak of activity, the pool of cyclin E-cdk2 complexes free of cdk inhibitors represented only a minor proportion of the total cyclin E-cdk2 complexes present in the MCF-7 cells.

To test if the observed cyclin E-cdk2 activity was associated exclusively with the cyclin E-cdk2 complexes that were free of p21 and p27, we evaluated the cyclin E-dependent kinase activity remaining in the supernatants of the extracts that were previously immunodepleted with p27 antibodies either alone or in conjunction with p21 antibodies (Fig. 8, top). As control, we assayed the cyclin E-dependent kinase activity left in the supernatant fractions after cyclin E immunodepletion. The results of histone H1 kinase assays after immunoprecipitation of these immunodepleted extracts with cyclin E antibodies are shown in Fig. 8 (bottom). As expected, cyclin E immunodepletion completely removed all cyclin E-dependent kinase activity from the extracts (lanes 7 and 8). However, neither p27 immunodepletion nor simultaneous immunodepletion of p21 and p27 decreased the cyclin E-dependent kinase activity of the extracts significantly compared to mock-depleted extracts (lanes 1 to 6). Thus, we concluded that the active cyclin E-cdk2 complexes formed after estradiol-induced cell cycle progression are devoid of these two cdk inhibitors.

Taken together, these results argue that estradiol was able to cause a reduction in the levels of cdk-inhibitory activity associated with cyclin E-cdk2 complexes during the tamoxifen arrest. This reduction was achieved through the induction of cyclin D1 expression, which allowed the formation of cyclin D1-cdk4-cdk6 complexes; these complexes, in turn, served to abstract p21 associated with cyclin E-cdk2, giving rise to active cyclin E-cdk2 complexes.

DISCUSSION

The studies presented here describe the effects that estrogen has on the growth of estrogen-sensitive MCF-7 cells. We conducted these experiments with the aim of determining the specific mechanisms by which estrogen affects the cell cycle clock and leads thereby to the proliferation of mammary epithelial cells. Our data indicate that estrogen can induce rapid and strong activation of cyclin E-cdk2 complexes through its ability to increase cyclin D1 expression. The observed rapid response of the cell cycle clock apparatus to estrogen stimulation argues for a direct effect of estrogen on one or more components of this apparatus. Conversely, it provides evidence against an indirect mechanism involving induction by estrogen of growth factors that in turn act in an autocrine fashion to elicit the observed responses.

The upregulation of cyclin E-cdk2 activity took place as a consequence of the increased cyclin D1 expression induced by estrogen. The increased levels of cyclin D1 led to increased cyclin D1-cdk4 complexes (2, 40), a resulting increase in the amount of the p21 cdk inhibitor associated with this cyclin-cdk complex, and a corresponding reduction in the amounts of p21 bound to cyclin E-cdk2 complexes. This redistribution of the p21 inhibitor then permitted activation of the cyclin E-cdk2 complexes. Similar models have been proposed to explain the mechanism of action of growth inhibitors. For example, TGF-β, by inducing expression of p15 or by downregulating cdk4, can lead to a redistribution of the p27 cdk inhibitor, from cyclin D1-cdk4 to cyclin E-cdk2 complexes (11, 16, 43).

Role of cyclin D1 protein in proliferation of ER-positive cells. Cyclin D1 gene amplification is observed in 15 to 30% of breast cancers (39). A strong correlation of increased levels of cyclin D1 mRNA with ER overexpression has also been noted elsewhere (7). Moreover, this amplification has been associated with a poor prognosis for ER- and progesterone receptor-positive breast tumors (5). Our results indicate that estrogen can induce expression of cyclin D1 in the first hours after its addition to tamoxifen-arrested cells (Fig. 3A and 7A). Thus, these results support the view that estrogen affects expression of cyclin D1 directly.

The model presented here suggests that the ER and cyclin D1 conspire to drive human mammary carcinoma cells through the G_1 phase of their cell cycle. High levels of the ER can cause high expression of cyclin D1 and the resulting removal of p21 from cyclin E-cdk2 complexes. Alternatively, high levels of cyclin D1, which may result from amplification of the cyclin D1 gene or other ER-independent mechanisms, may achieve the same end. Moreover, tumors expressing high levels of either the ER or cyclin D1 may be able to overcome the inhibitory

effects of concomitantly expressed p21 by sequestering the latter in cyclin D1-cdk4 or cyclin D1-cdk6 complexes. Our model is further supported by the work of Musgrove et al. (34). These authors have developed an inducible cyclin D1 system using another ER-positive breast cancer cell line (T47D). Induction of cyclin D1 expression in these cells leads to activation of cyclin E-cdk2 kinase, pRb hyperphosphorylation, and cell cycle progression.

Induction of cyclin D1 by estrogen recruits p21. The present data indicate that the cdk inhibitor p21 serves to couple cyclin D1 levels with the activity of cyclin E-cdk2. We conclude that p21 is involved in the observed cyclin E-cdk2 activation based on the following facts. (i) Extracts obtained from tamoxifen-arrested cells contain a readily detected cdk2-inhibitory activity (Fig. 6A). (ii) This activity can be immunodepleted by p21-specific antibodies but not by anti-p27 antibodies (Fig. 6C). (iii) The ability of p21 antibodies to immunodeplete cyclin E decreases after estradiol treatment of tamoxifen-arrested cells (Fig. 7B, bottom). (iv) Immunodepletion of cyclin D1 removes most of the p21 from extracts of estradiol-stimulated cells but not when extracts from tamoxifen-arrested cells are used (Fig. 7B, top). (v) Levels of cyclin D1 that are associated with p21 increase substantially following estrogen-mediated release of the tamoxifen block (Fig. 7A).

While this work was in progress, Foster and Wimalasena (13), using methionine-glutamine-deprived MCF-7 cells, also observed increased synthesis of cyclin D1, cyclin E-cdk2 activity, and pRb hyperphosphorylation after estradiol-induced cell cycle progression. However, these authors propose that the regulation of cyclin E-cdk2 activity is due to a decrease in p27. The slower kinetics of cyclin E-cdk2 activation and the changes in p27 levels observed by these authors may be due to their use of amino acid starvation to synchronize MCF-7 cells.

Our model of cyclin D1-mediated p21 redistribution is further supported by biochemical measurements that gauge the relative affinities of the p21 cdk inhibitor for association with the cyclin E-cdk2 and cyclin D2-cdk4 complexes. Others have shown elsewhere (17) that the K_i value for inhibition of cyclin E-cdk2 by p21 is 3.7 nM while that for cyclin D2-cdk4 is 0.6 nM. We presume that these inhibitory concentrations reflect the relative affinities of p21 for these two types of complexes and that the affinity of p21 for cyclin D2-cdk4 is similar to that for cyclin D1-cdk4. These relative affinities of p21 would therefore explain the ability of cyclin D1-cdk4 or cyclin D1-cdk6 complexes to abstract p21 from cyclin E-cdk2 complexes. Consistent with this notion is the recent demonstration that cyclin D1 is more effective than cyclin E in rescuing p21-dependent growth suppression (28).

Yet other lines of evidence point to a role played by cyclin D-cdk4 or cyclin D-cdk6 complexes as a reservoir of cdk inhibitors (41, 46, 48). For example, the levels of different cdk inhibitors relative to those of cyclin D-cdk4 or cyclin D-cdk6 complexes may determine a signal threshold level that determines the timing in G_1 of cyclin E-cdk2 activation and consequent pRb phosphorylation. Consistent with this reservoir model, others have shown that p53-mediated growth arrest through induction of p21 can be overcome by ectopically expressed, inactive forms of cdk4 and cdk6 (27).

The dramatic increase of cyclin E-cdk2 catalytic activity following estradiol treatment is due to the translocation of p21 from the cyclin E-cdk2 complexes to cyclin D1-cdk4 complexes. Perhaps surprisingly, this marked increase in the functioning of the cyclin E-cdk2 complexes is attributable to the activity of only a small minority of the complexes present in the estrogen-treated cells. Thus, after estradiol treatment, the entirety of the cyclin E-cdk2 catalytic activity is traceable to a small proportion of the cyclin E-cdk2 complexes that have been freed of p21; the vast majority of these complexes remain associated with cdk inhibitors and in a functionally inactive state. Therefore, in contrast with previous reports (17, 55), we find that the dissociation of p21 from cyclin E-cdk2 complexes is essential for their functional activity. A similar requirement for the removal of p21 from cdk2 complexes has been noted recently, arguing that p21 interaction is strictly inhibitory for cyclin E-cdk2 complexes present in certain cells (27).

In summary, we conclude that estrogen, by regulating cyclin D1 expression and p21 distribution, can control cyclin E-cdk2 activity and pRb phosphorylation in breast cancer-derived MCF-7 cells. This major role of estrogen in controlling levels and activities of G_1 cyclin and the associated kinases fits well with its essential role in driving mammary epithelial cell proliferation.

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A Paracrine Role for the Epithelial Progesterone Receptor in Mammary Gland Proliferation and Differentiation

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Abstract

Recently generated progesterone receptor-negative ($PR^{-/-}$) mice provide an excellent model for dissecting the role of progesterone in the development of the mammary gland during puberty and pregnancy. As $PR^{-/-}$ mice do not exhibit estrous cycles and consequently fail to become pregnant, the full extent of the mammary gland defect in these mice caused by the absence of the PR can not be assessed. However, by transplanting wildtype (wt) mice with $PR^{-/-}$ breasts, we demonstrate that the development of the mammary gland in the absence of the PR is arrested at the stage of a simple ductal system found in the young virgin mouse.

Mammary transplants lacking the PR in the stromal compartment give rise to normal alveolar growth while transplants containing $PR^{-/-}$ epithelium conserve the abnormal phenotype. Chimeric epithelia in which $PR^{-/-}$ cells are in close vicinity to PR wt cells go through complete alveolar development to which the $PR^{-/-}$ cells contribute. Together, these results indicate that progesterone acts by a paracrine mechanism on a subset of mammary epithelial cells to allow for alveolar growth and that expression of the PR is not required in all the cells of the mammary epithelium in order for alveolar development to proceed normally.

Introduction

The mouse provides a useful model to study mammary gland development. At the onset of puberty, a simple system of branching ducts begins growing out from the nipple area into a pad of fatty connective tissue that underlies the skin. During the luteal phase of the estrous cycles, the ductal system becomes more complex through the growth of sidebranches. Ductal sidebranching becomes more extensive during early pregnancy and subsequently alveolar bodies develop from these ducts, fill up the fat pad, and differentiate to become the sites of milk production.

The serum levels of the sex steroid progesterone are elevated during diestrous, the phase of luteal activity of the estrous cycle, and pregnancy. Moreover, experimental manipulation of the hormonal system has implicated this hormone as an essential stimulus required for the induction of ductal branching and for alveologenesis (Nandi, 1958). However, the elucidation of the role of progesterone is complicated by the fact that in the mammary epithelium, synthesis of the progesterone receptor (PR) depends on estrogen, the serum levels of which are also elevated during puberty and pregnancy. This has made it difficult to assess what effects can be attributed to progesterone alone.

In order to dissect the role of progesterone from that played by estrogen, we generated mice lacking the PR by targeted inactivation of the PR gene in the mouse germline (Lydon et al., 1995) . The mammary glands of the resulting young virgin $PR^{-/-}$ females show the same extent of ductal development as is seen in wt female mice (Lydon et al., 1995). However,

when wt and $PR^{-/-}$ virgin females were exposed to estradiol and progesterone, the wt breast tissue responded with sidebranching and lobuloalveolar development while the mammary glands of $PR^{-/-}$ females remained essentially unchanged. This suggested that PR is not required for initial ductal growth but is essential for subsequent sidebranching and alveologenesis.

The administration of exogenous estrogen and progesterone, as was done in the above-described experiments and in a subsequent study extending this work (Humphreys et al., 1997) did not permit us to properly gauge the full contributions of the PR to mammary development. Thus, such administration does not reproduce the full spectrum of complex hormonal changes that occur during a normal pregnancy. During this period, the serum levels of a wide array of other hormones including growth hormone, prolactin, placental lactogen, and adrenal steroids are elevated. Moreover, the secretion of each of these hormones follows specific diurnal rhythms, and it is unlikely that injections of exogenous hormones achieve physiologic serum levels and correct local concentrations.

For these reasons, we resorted to transplanting $PR^{-/-}$ mammary tissues into wt animals that were subsequently impregnated. This allowed us to study the morphogenesis of the breast tissue in a hormonal environment that faithfully recapitulated that seen in pregnant, unmanipulated, wt animals. The results of previous research did not provide us with clear predictions of the outcomes of these transplantation experiments. For example, the PR is expressed in both stromal and epithelial compartments of the mammary gland (Haslam and Shyamala, 1981). Within the epithelium, the distribution of the PR is variegated (Silberstein et al., 1996). Together, such observations provided no clear indication of the contributions of

various subtypes of stromal and epithelial cells to mammary epithelial morphogenesis occurring in the presence or absence of the PR.

By grafting $PR^{-/-}$ epithelium or stroma in combination with PR wt stroma or epithelium, we have found that the primary target for progesterone is the mammary epithelium while a direct response of the mammary stroma is not required in order for sidebranching and lobuloalveolar development to occur. Furthermore, $PR^{-/-}$ mammary epithelial cells can give rise to alveoli when placed in close vicinity to PR wt epithelial cells, indicating that progesterone does not need to act directly on the alveolar cells and instead can orchestrate the morphogenetic and proliferative events of alveologenesis by affecting nearby cells in the mammary epithelium.

Results

Development of the mammary gland during pregnancy in the absence of the PR

In order to analyze the role progesterone plays in the mammary gland during normal pregnancy, entire mammary glands from $PR^{-/-}$ female mice and their wt littermates were transplanted onto the abdominal muscle wall of PR wt females. The transplanted glands included both epithelial and stromal compartments. The recipient females were of the same 129SV/C57Bl6 genetic background and were homozygous for the inactivated RAG1 allele (Mombaerts et al., 1992). Females of this genotype are immunocompromised and therefore able to accept allografts. The

engrafted females were mated three weeks after surgery and sacrificed immediately after a completed pregnancy. In all cases, the implants along with an endogenous mammary gland were analyzed by wholemount microscopy.

While the wt implants and endogenous glands (Fig.1, central and bottom panel respectively) showed full alveolar development at parturition, the $PR^{-/-}$ grafts developed only a simple ductal system (Fig. 1, top panel). These observations validated the transplantation procedure. More significantly, they demonstrated, as suggested by previous reports (Lyons, 1958; Nandi, 1958), that progesterone is essential for sidebranching and lobuloalveolar growth and showed that, in the absence of the PR, the mammary gland fails to undergo substantial proliferation in the presence of the full array of pregnancy-associated hormones.

Involvement of the stromal and the epithelial compartments in PR-mediated responses

To address the question of whether progesterone acts on the mammary stroma or epithelium, engrafted animals were created in which either the mammary epithelium or the fat pad lacked PR due to inactivation of the PR gene. The development of the mammary gland in response to physiological hormonal stimulation was then followed.

In the mouse, the mammary epithelium grows out from the nipple into a fatpad that underlies the skin. At three weeks after birth, the epithelium of the gland has not yet penetrated extensively into the stroma and can be eliminated by removing the nipple region of the mammary gland

(DeOme, 1959). Mammary epithelial cells (MECs) that are introduced into the remaining "cleared" fat pad will give rise to a new ductal system. They can grow out from a piece of breast tissue that is placed into the fat pad (DeOme, 1959) or from single-cell suspensions that are injected into the fat pad (Daniel and DeOme, 1965).

We adapted these surgical procedures to create mammary glands that specifically lacked the PR in their stromal cells. Briefly, the nipple regions containing the mammary epithelium were removed from the fourth mammary glands of 3-week old $PR^{-/-}$ females and their wt littermates. The resulting cleared fat pads were then implanted with mammary epithelium derived from a wt donor. Subsequently, the resulting reconstituted mammary glands were dissected and transplanted onto the abdominal muscle wall of $RAG1^{-/-}$ females.

We validated this transplantation procedure by implanting PR wt epithelium into PR wt fatpads. The resulting engrafted glands developed like the endogenous mammary glands in virgin as well as post partum recipients, demonstrating that the engrafted fat pad had become fully vascularized when transplanted in this fashion. To rule out any necessary contributions of fibroblasts that may have been carried along with the implanted epithelial cells, we injected the fat pads with a suspension of epithelial cells derived from pure epithelial cultures (Kittrell et al., 1992) as assessed by keratin stain (data not shown) and confirmed that even in this case, fully developed mammary glands could be obtained.

The interpretation of these experiments depended upon our ability to distinguish implanted mammary epithelium from any residual endogenous epithelium that inadvertantly had not been removed during the preparation of the cleared mammary fat pads. In fact, in the virgin gland, it is easy to

distinguish ducts arising from implanted epithelium from those that are endogenous to this gland because of the distinctive orientations of ductal growth. Thus, the endogenous epithelium grows unidirectionally from the nipple into the fat pad while the ducts arising from the implant, which we place into the center of the cleared fat pad, grow centrifugally. At parturition, however, when the fat pad is filled with alveoli, it is difficult to distinguish the two ductal systems, making it impossible to rule out that the observed epithelial structures derive from residual endogenous epithelium that has been left behind during the preparation of the cleared fat pad.

To address this difficulty, mammary epithelium derived from ROSA26 female mice was exploited (Friedrich and Soriano, 1991). Mice of this transgenic strain, which is of 129SV/C57Bl6 genetic background, express the β-galactosidase gene in virtually all their tissues. The mammary epithelium of these ROSA26 mice was implanted into the cleared fat pads of wt mice. When these reconstituted fat pads were subjected to an X-gal staining procedure, the implanted ROSA26-derived epithelium turned blue and could thus be unequivocally distinguished from any endogenous epithelium inadvertantly left behind, which was visualized by the red color of the carmine alum counterstain. Together, the above-described preliminary experiments and the use of ROSA cells validated our transplantation procedures and our ability to study engrafted tissues without the confounding effects of residual tissue originating from the recipient breast.

The above procedures were utilized to resolve the respective roles of stromal and epithelial derived PR populations in mammary gland proliferation and differentiation. First, ROSA26.PR wt epithelium was transplanted into cleared $PR^{-/-}$ fat pads; the resulting reconstituted

mammary glands were then placed onto the abdominal muscle wall of a RAG1$^{-/-}$ recipient female. Four weeks later, the engrafted RAG1$^{-/-}$ recipients were mated. After they had given birth, the transplanted mammary gland as well as an endogenous mammary gland were analyzed by wholemount microscopy. As can be seen in Figure 2, the injected ROSA26-derived mammary epithelial cells grew equally well in transplanted fatpads from wt (bottom panel) and PR$^{-/-}$ (top panel) donors. This demonstrated that the presence of the PR in the mammary stroma was not essential for the pregnancy-induced sidebranching and lobuloalveolar development.

Next the role of the PR in the epithelium independent of its function in the stroma was assessed. To do this, mammary epithelial cells derived from either PR$^{-/-}$ or wt donors were transplanted into the cleared mammary fat pads of wt recipients. The engrafted recipients were mated and their mammary glands analyzed at parturition. The results of these experiments are shown in Figure 3. Whereas the wt implant gave rise to a fully developed mammary tree, the epithelium lacking the PR only grew into a simple ductal tree (Fig. 3, left panels). Similarly, when we analyzed the mammary glands of engrafted virgin females two months after surgery, the wt implant as well as the endogenous breasts showed sidebranching whereas the PR$^{-/-}$ breast had only a simple ductal system (Fig.3, right panels). Table 1 summarizes the results of these transplantation experiments. These results allowed us to conclude that the mammary epithelium is the prime target of progesterone both before and during pregnancy, and that a direct response of the mammary stroma to progesterone does not play an essential role in lobuloalveolar development.

Role of the PR in the proliferation of alveolar cells

The experiments above indicated that the absence of the PR from all cells of the mammary epithelium resulted in a failure of sidebranching and lobuloalveolar growth. However, they did not address the question of whether the presence of PR was required in all cells of the ductal epithelium or only in a subset of MECs in order for these morphogenetic processes to proceed normally.

To distinguish between these possibilities, we created mosaic mammary epithelia containing both $PR^{-/-}$ and $PR^{+/+}$ MECs. The latter cells were derived from ROSA26 mice. In this case, tissue structures composed of $PR^{+/+}$ cells would turn blue upon X-gal stain when analyzed by wholemount microscopy. Structures composed of $PR^{-/-}$ cells would turn red, being stained only by the carmine alum counterstain.

Mixtures of $PR^{+/+}$ and $PR^{-/-}$ MECs were injected into the cleared mammary fat pads of $RAG1^{-/-}$ females. These mixtures were obtained either by combining single cell suspensions derived from $PR^{-/-}$ and $PR^{+/+}$.ROSA26 primary cultures or by mixing finely minced mammary tissues dissected from females of these two strains. Two months later, the engrafted recipients were mated and the engrafted breasts were analyzed post partum.

Depending on the degree of homogeneity of the injected mixture, we found two types of chimerism. In the first type, which arose mostly from injected minced tissue, the mammary glands showed discrete sectors having distinct phenotypes. An example, representative of 17 samples of this type of chimerism, is shown in Figure 4B. One half of the epithelial component

of the mammary gland stained red while the other half stained blue; this indicated the origins of these two sectors from $PR^{-/-}$and ROSA26 engrafted cells respectively. The sector comprising $PR^{-/-}$ cells represents a simple ductal tree while the sector composed of the $PR^{+/+}$.ROSA26 cells shows extensive lobuloalveolar growth. This demonstrated that the coexistence of MECs of $PR^{+/+}$ and $PR^{-/-}$ in one fatpad is not sufficient to rescue the morphogenetic defect intrinsic to the $PR^{-/-}$ cells.

Most of the chimeric epithelia that arose from mixed single-cell suspensions, which allowed for very homogeneous mixing of the two MEC types, showed complete lobuloalveolar development (Fig. 4A). However, at higher magnification distinct red alveoli and blue alveoli could be identifed (Fig. 4A'). This suggested but did not prove that $PR^{-/-}$ cells could participate in alveolar formation if they were in close proximity with wildtype MECs.

Any conclusions concerning the ability of the $PR^{-/-}$ MECs to form alveoli were clouded by the possibility that certain $PR^{+/+}$.ROSA cells that participated in alveologenesis had failed to stain blue, thereby taking on the appearance of the $PR^{-/-}$ cells in the same mixed grafts. To address this issue, we crossed the β-galactosidase transgene into the $PR^{-/-}$ genetic background. Subsequently, suspensions of $PR^{-/-}$ROSA26 MECs were mixed with $PR^{+/+}$ MECs lacking the β-galactosidase transgene to generate chimeric breasts. On this occasion, we looked for the opposite result to that seen previously - alveolar cells that stained blue. Indeed, as shown in Fig. 5, a representative of 26 independent grafts, the mammary glands obtained from pregnant engrafted females showed areas with blue alveoli, proving conclusively that $PR^{-/-}$ cells can participate in the formation of alveoli if they are in close vicinity to wt epithelial cells. (The results are summarized

in table 2.) This indicated, in turn, that the presence of the PR is required in only a portion of the MECs in order for lobuloalveolar development to occur. Moreover, it suggested that progesterone activates on a paracrine signaling route that operates between distinct subtypes of MECs and helps to drive lobuloalveolar proliferation.

Discussion

Hormonal ablation/reconstitution experiments (Nandi, 1958) have suggested that progesterone plays an important role in the changes that the mammary gland undergoes during early pregnancy, namely sidebranching and initial alveolar growth. To determine the extent to which progesterone signaling is limiting in development, we generated mice lacking the PR gene (Lydon *et al.*, 1995). However, because the $PR^{-/-}$ females have multiple impairments in their reproductive functions, the specific consequences of PR inactivation on mammary gland development could not be assessed in these mice.

To circumvent this difficulty, we have used various transplantation techniques to elucidate the role of progesterone in the development of the mammary gland. In particular, we have made use of cells derived from mice carrying the β-galactosidase transgene. These cells turn blue upon X-gal staining, making it possible to distinguish these cells histochemically from neighboring β-galactosidase-negative cells. In one experiment, this allowed us to distinguish the β-galactosidase-positive implanted MECs from the β-galactosidase-negative endogenous cells of an engrafted breast; in

another setting, this procedure made it possible for us to distinguish MECs carrying two functional PR alleles from those lacking the PR.

Most transplantation experiments involving non-syngeneic grafts have exploited nude mice as recipients. We note here in passing the utility of the RAG1$^{-/-}$ mice used for transplantation experiments designed to elucidate mammary gland physiology. Because nude mice have low estrogen levels, they do not represent good recipients in transplantation experiments designed specifically to this purpose. In contrast, the RAG1$^{-/-}$ mice used here exhibit developmental defects that are strictly limited to B- and T-cell development.

Our initial experiments involving the transplantation of PR$^{-/-}$ mammary glands into PR$^{+/+}$.RAG1$^{-/-}$ females were motivated by the need to assess the role of the PR in an *in vivo* physiologic environment in which the full array of pregnancy-associated hormonal signals was present. PR$^{-/-}$ mammary glands grafted to a PR$^{+/+}$.RAG1$^{-/-}$ recipient developed only a simple ductal system, even when the host went through a series of estrous cycles and a normal pregnancy. This indicated that sidebranching and lobuloalveolar growth rely on the presence of the PR and that other signaling mechanisms operating in the breast tissue cannot compensate for the absence of the PR to allow these processes to proceed normally.

These initial results left us with two distinct scenarios. In one, both sidebranching and lobuloalveolar proliferation, each in its own right, depends on the presence of progesterone. In the other, sidebranching is dependent on progesterone while lobuloalveolar growth depends on prior sidebranching and is therefore only indirectly dependent on progesterone. Our analysis of a series of wholemounts of mammary glands from wt pregnant mice showed that alveoli sprouted not only from sidebranches

(secondary ducts) but also from the primary ducts (data not shown). This indicated that sidebranching is not an absolute prerequisite for alveolar growth. For this reason, we concluded that the PR is required for lobuloalveolar proliferation *per se* in addition to its demonstrated role in sidebranching.

We next addressed the issue of whether progesterone needs to act on the mammary stroma, the epithelium, or both. One important clue for resolving this puzzle appeared to come from the longstanding observation that morphogenesis in many epithelial-mesenchymal organs such as the mammary gland is controlled by inductive events (Grobstein, 1955) that require cross-talk between epithelial and stromal components. In the breast in particular, the embryonic mammary mesenchyme induces the overlying epithelium to develop into the mammary bud (Propper, 1968). Moreover, in male embryos of various mouse strains, androgens induce the involution of the mammary anlage. Using wt and androgen-insensitive *Tfm (testicular feminization)* mutant mice to generate chimeric epithelial-mesenchymal tissue combinations, others have demonstrated that testosterone acts on the mammary stroma to induce this process (Kratochwil and Schwartz, 1976; Drews and Drews, 1977).

Several lines of evidence indicate that the stroma is also important in mediating ductal growth in response to estrogen. *In vitro*, estrogen can only exert a growth-stimulatory effect on primary mammary epithelial cells (MECs) if mammary fibroblasts are present (Haslam, 1986; Haslam and Levely, 1985, McGrath, 1983). Indeed, the cells that actively divide during ductal growth (cap cells) are ER-negative whereas stromal cells in their vicinity stain ER-positive (Daniel *et al.*, 1987). In addition, when castrated

virgin mice are injected with estradiol, DNA synthesis in the mammary stroma precedes that in the epithelium (Shyamala and Ferenczy, 1984).

The role of the stroma in mediating progesterone-dependent processes in the breast has been less clear. For example, ligand binding studies have shown that 80% of the progesterone receptors in the mouse mammary gland localize to the epithelium while the remaining 20% are found in the stroma (Haslam and Shyamala, 1981). Such observations have been compatible with models in which the epithelial cells, the stromal cells, or both cell types are required to mediate the direct responses to progesterone.

More recently, epithelial/stromal reciprocal transplantations between wt and $ER^{-/-}$ and wt and $PR^{-/-}$ tissues, have demonstrated that stromal derived ER and PR exert paracrine effects on the epithelium both in the uterus (Cooke et al., 1997) and in the vagina (Cunha and O'Malley, unpublished observations). We show here that mammary glands lacking PR in the stroma undergo normal development while the absence of the PR from the epithelium confers the $PR^{-/-}$ phenotype, indicating that the target cells of progesterone in the mammary gland are the epithelial cells. While effects of progesterone on the mammary stroma can not be excluded, they do not appear to contribute in any obvious way to the development of the ductal tree and alveoli.

Recently reported experiments in which we participated (Humphreys et al., 1997). yielded results that are in conflict with one aspect of the present work. These previous experiments appeared to indicate that the PR that functions within the stromal compartment exerts an effect on epithelial ductal growth, contrary to the present results which indicate the opposite. We find the present results more compelling for several reasons. The

number of transplanted animals examined here was much larger. Moreover, we have analyzed the behavior of mammary glands in a situation in which the only PR-negative tissue in engrafted animals was the mammary stroma; the earlier work, in contrast, examined the behavior of wild-type epithelium transplanted into the cleared $PR^{-/-}$ fat pad of a $PR^{-/-}$ host.

The present work together with previous observations of others (Lyons, 1958; Nandi, 1958) indicate that progesterone is required for two distinct morphogenetic processes in the breast - sidebranching and preparation of ductal cells for subsequent lobuloalveolar development. The precise mechanisms by which progesterone enables ductal MECs to participate in alveologenesis has been unclear. The pattern of PR expression in the mammary epithelium is inhomogeneous (Silberstein *et al.* 1996), suggesting the involvement of only a subset of ductal cells in progesterone-triggered processes. The connected issue of whether the PR-expressing cells represent the precursors of the alveolar outgrowths is addressed here.

Our observation that $PR^{-/-}$ cells can give rise to alveolar structures if they are in close vicinity to $PR^{+/+}$ cells indicates that progesterone does not need to act directly on a ductal epithelial cell in order for it to participate in alveolar formation. Instead, it appears that progesterone acts on a subtype of ductal cell, causing it to release paracrine signals that permit other nearby epithelial cells to participate directly in lobuloalveolar proliferation.

The present work provides no indication about the nature of the paracrine signal released by the progesterone-activated ductal cell. However, the observation that very close apposition of PR-positive with PR-negative cells is required to rescue the $PR^{-/-}$ phenotype indicates that the signal, whatever its biochemical nature, is transmitted only over short intercellular distances and is unlikely to be freely diffusible. Factors that

are tightly associated with the extracellular matrix such as wnt proteins and FGFs, which are differentially expressed during mammary gland development (Coleman-Krnacik and Rosen, 1994; Gavin and McMahon, 1992) , are attractive candidates for conveying such paracrine signals. Our data provide no indication whether or not these paracrine signals communicate directly between the progesterone-activated ductal cells and closely apposed alveolar precursor cells. It remains equally possible that the progesterone-activated ductal cell communicates with the stroma; the latter, in turn, may pass on a signal directly to the alveolar precursor cells as suggested by others (Birchmeier and Birchmeier, 1993). The use of tissue reconstitution techniques and genetically altered cells should allow the further dissection of the molecular mechanisms of mammary morphogenesis over the next several years.

Materials and Methods

Mice

ROSA26 and RAG1$^{-/-}$ mice were purchased from Jackson Laboratories. For PR genotyping, genomic DNA was isolated from tails and analyzed by PCR. PCR was performed by denaturing the DNA at 94 ^{0}C for 1 min, followed by 30 cycles of amplification: 94 ^{0}C for 1 min, 60 ^{0}C for 2 min, 72 ^{0}C for 1 min, and a final extension step at 72 ^{0}C for 5 min. The following PR specific primers were used *P1*(5'-TAG ACA GTG TCT TAG ACT CGT TGT TG-3'), *P2*(5'-AGC AGA AAA CCG TGA ATC TTC-3'), and a *neo* gene-specific primer, *N* 2(5'-GCA TGC TCC AGA CTG CCT TGG GAA A-3').

Presence of the β-galactosidase transgene was tested for by subjecting a piece of tail to the X-gal stain procedure described below.

Whole Breast Transplant

4 to 6-week-old $PR^{+/+}$ or $PR^{-/-}$ female mice were sacrifced and their inguinal mammary glands were dissected. $RAG1^{-/-}$ females of the same age were anesthetized with Avertin i.p. (Hogan et al., 1995). The ventral skin was incised and the abdominal muscle wall exposed. A $PR^{-/-}$ and a $PR^{+/+}$ mammary gland were placed onto the abdominal wall and the incision was closed using surgical staples. Three weeks after surgery the recipients were mated. They were sacrificed at parturition. The two transplanted glands and an endogenous mammary gland were analyzed by wholemount microscopy.

Fat Pad Transplant

3-week old $PR^{+/+}$, $PR^{+/-}$ and $PR^{-/-}$ females were sacrificed and their inguinal mammary glands were exposed. The nipple-near region was removed. Into the remaining empty fat pad we injected primary mammary epithelial cells derived from ROSA26 females. The engrafted fat pads were placed onto the abdominal muscle wall of virgin $RAG1^{-/-}$ females.

Transplantation of Mammary Epithelium

The fat pads of 3-week-old $RAG1^{-/-}$ females were cleared (see above). Pieces of mammary tissue of 1mm diameter were removed from the nipple region of $PR^{+/+}$ and $PR^{-/-}$ females and implanted as described before (DeOme, 1959) Alternatively, the cleared fat pads were injected with $PR^{+/+}$ and $PR^{-/-}$ primary cells, see (Daniel and DeOme, 1965).

Cell Culture

For the culture of primary mouse MECs we followed the procedure estblished by Medina (Kittrell et al., 1992).

Mammary Gland Whole Mounts

The inguinal mammary glands were dissected, spread onto a glass slide, fixed in a 1:3 mixture of glacial acetic acid: 100% ethanol, hydrated, stained overnight in 0.2% carmine (Sigma) and 0.5% AlK(SO4)2, dehydrated in graded solutions of ethanol, and cleared in BABB (benzyl alcohol and benzylbenzoate (1:2), Sigma) as described previously (Wang et al., 1990). Pictures were taken on a Leica MZ12 stereoscope with Kodak Ektachrome 160T.

X-gal stain

The transplanted mammary glands were dissected, fixed for an hour in 4% formaldehyde in phosphate-buffered saline (PBS), washed three times over 3 hours with rinse buffer (2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% NP40 in PBS) and rotated in X-gal staining solution (1 mg/ml X-gal (5-Bromo-4-chloro-3-indoyl-β–D-galactopyranoside), 5 mM K-ferricyanide, 5 mM K-ferrocyanide in rinse buffer) at 37 ^{o}C for 18 hours, washed in PBS and processed for wholemounting as described above.

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Figure 1

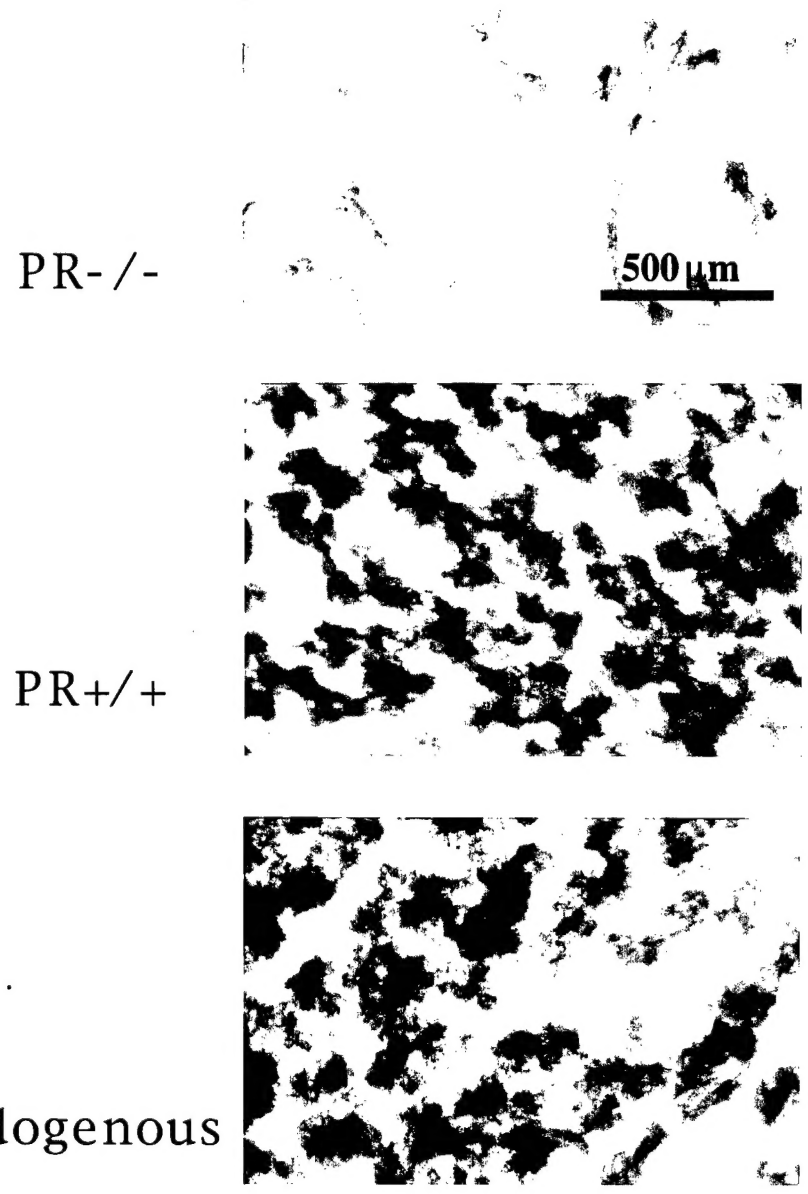

Figure 2

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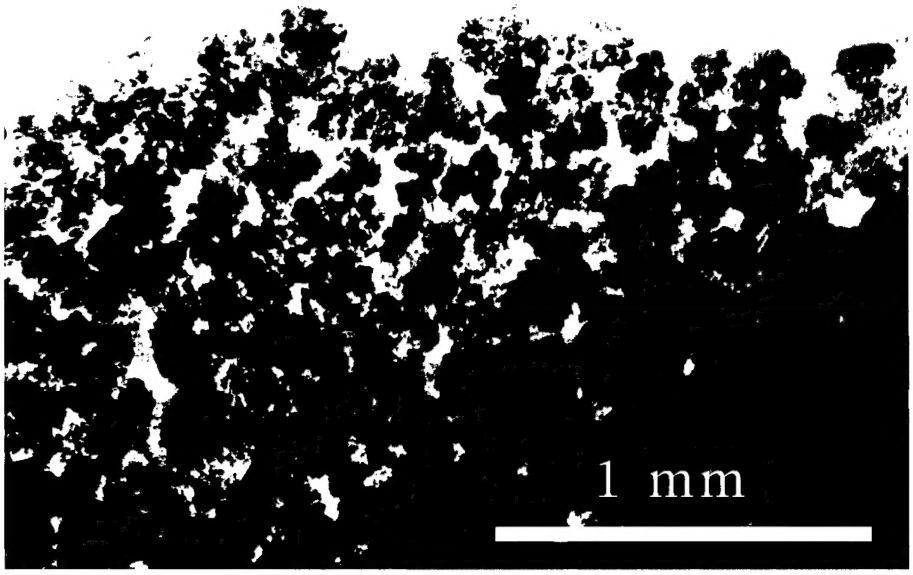

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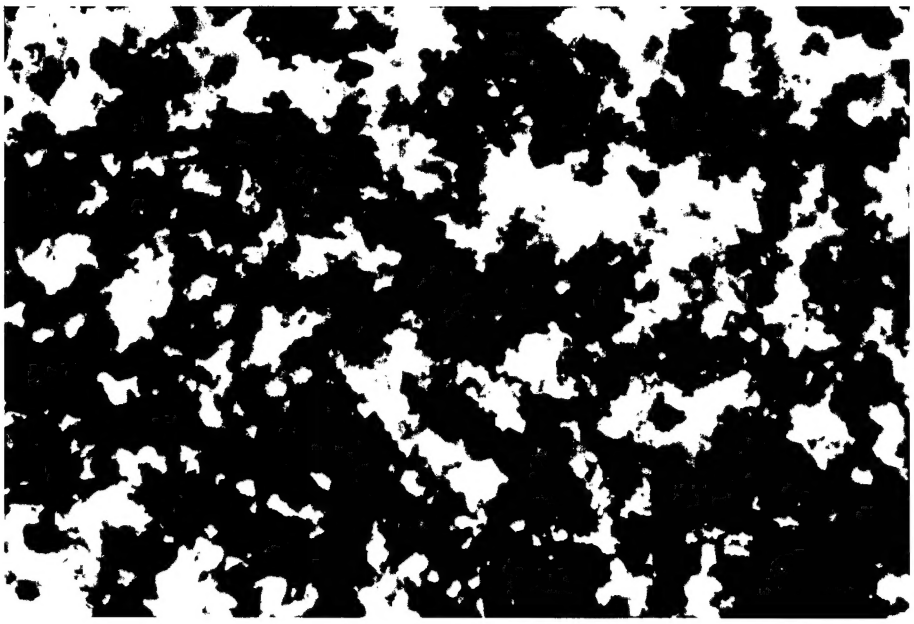

Figure 3

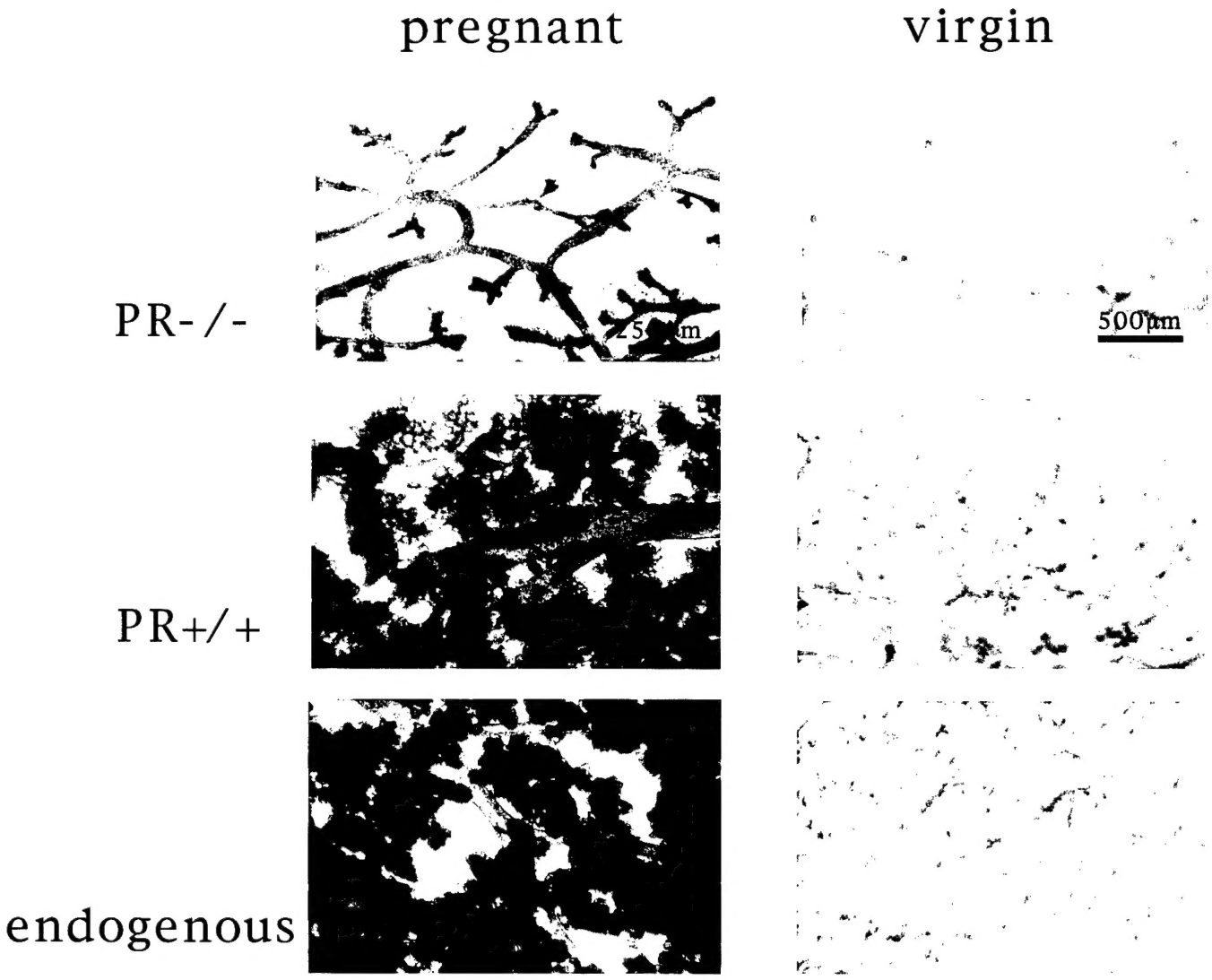

Figure 4